Analysis of HCV Coinfections among Newly Diagnosed HIV Cases in Germany

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DEDICATION

This dissertation is dedicated to my:

- ♥ Family, especially to my father Eshetu (አበባ), for the never-ending love and support.
- ♥ Late mom, Belaynesh (እማ), for the love and sacrifice beyond measure.
- ♥ So beloved wife, Tigest (ማሬ), for the advice, patience and endless support.
- ♥ Lovely daughter, Mayra (ማይራ), for bringing joy into my life.

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LIST OF ABBREVIATIONS

AASLD	American Association for the Study of Liver Diseases
Ab	Antibody
Ag	Antigen
ALT	Alanine transaminase
ART	Antiretroviral therapy
BSA	Bovine Serum Albumin
bp	Base pair
C	Core
CDC	Centers for Disease Control and Prevention
cDNA	Complementary Deoxyribonucleic Acid
CKD	Chronic Kidney Disease
DAA	Direct Acting Antiviral
DBS	Dried Blood Spot
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
DSS	Dried Serum Spot
DS/PS	Dried Serum/Plasma Spot
DTT	Dithiothreitol
EIA	Enzyme Immunoassay
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic Reticulum
FDA	Food and Drug Administration
FDC	Fixed Dose Combination
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
IDU	Injection Drug Users
IgG	Immunoglobulin G
IU	International Units
MSM	Men who have sex with men
nM	Nanomolar

NGS	Next-generation Sequencing
NIHCDC	National Institutes of Health Consensus Development Conference
ORF	Open Reading Frame
OST	Opioid Substitution Therapy
PCR	Polymerase Chain Reaction
Peg-IFN	Pegylated Interferon
PI	Protease Inhibitor
PrEP	Pre-exposure Prophylaxis
PWID	People Who Inject Drugs
RAS	Resistance-associated Substitution
RBV	Ribavirin
RNA	Ribonucleic Acid
RNasin	Ribonuclease Inhibitor
RKI	Robert Koch Institute
RT	Reverse Transcriptase
RT-qPCR	Quantitative Reverse Transcription PCR
St	Subtype
SVR	Sustained Virologic Response
TAE	Tris-acetate-EDTA
TMA	Transcription-mediated Amplification Assay
WHO	World Health Organization
5'UTR	5′ Untranslated Region
µM	Micro Molar

SUMMARY

In light of the major shift in HCV therapy resulting from the availability of highly potent direct acting antivirals (DAA), this study performed a surveillance of HCV coinfections among reported HIV new diagnoses in Germany. First the suitability of two commercial HCV ELISAs, Murex Ag/Ab (Abbott) and Monolisa Ag/Ab Ultra (Bio-Rad) for the detection of HCV in dried serum spots (DSS) was compared. The Murex ELISA was more sensitive in antigen positive plasma HCV seroconversion samples. In contrast, the Monolisa showed a higher sensitivity in HCV antibody positive DSS eluates. For this reason, samples from newly diagnosed HIV infections were examined using the Monolisa. Furthermore, an avidity test was established to distinguish between new and already longer existing infections. An optimal avidity index (AI) cut-off of 40% was determined for all genotypes, resulting in a period of 364 days for classification as new HCV infection. To distinguish active and resolved infections, positive samples were analyzed by RT-qPCR and Western blot. Genotyping and identification of resistance-associated substitutions (RAS) was performed by sequence analysis.

Of the total of 6,097 samples examined for the years 2015-2017, 396 were HCV ELISA reactive (6.5%). Of these, 256 (64.6%) were identified as active and 140 (35.4%) as resolved infections. A high proportion of HCV coinfections were observed in intravenous drug users (PWID, 77.8%, n=168/216), in the age group 30-39 years (9%, n=179/1,978) and in people of Eastern European origin (38.3%, n=124/324). Compared to 2016, the proportion of resolved infections in 2017 has increased in the total number of patients ($p < 0.01$) and especially in people of foreign ($p < 0.01$) and Eastern European ($p < 0.05$) origin. HCV-subtypes (St)-1a, St-3a, and St-1b were predominant with 33.9% (n=79/233), 33.5% (n=78/233) and 23.3% (n=52/233), respectively. In the samples from the diagnosis years 2015, 2016 and 2017, RAS were observed in a total of 69% (n=29/42), 60.8% (n=48/79) and 56.4% (n=22/39) samples, respectively. The proportion of St-1a and St-1b samples whose HCV sequence has resistance to DAAs was high in all diagnoses years (between 25% and 42.9%).

The observed increase in the proportion of resolved HCV coinfections can be attributed to DAA therapy. However, further efforts are needed to ensure that groups with continued high HCV prevalence benefit from this treatment. The detection of RAS in samples of St-1a and St-1b indicates a close surveillance to maintain the effectiveness of DAAs.

ZUSAMMENFASSUNG

In Anbetracht der enormen Verbesserung der HCV-Therapie durch die Entwicklung hochwirksamer und direkt wirkender Virostatika (DAA) dient diese Studie der Analyse von HCV-Koinfektionen unter den gemeldeten HIV-Neudiagnosen in Deutschland. Zunächst wurde die Eignung zweier kommerzieller HCV-ELISAs, dem Murex Ag/Ab (Abbott) und dem Monolisa Ag/Ab Ultra (Bio-Rad), zum Nachweis von HCV in getrockneten Serumspots (DSS) verglichen. Der Murex-ELISA zeigte sich sensitiver bei Antigen-positiven Plasma-HCV-Serokonversionsproben. Dagegen zeigte der Monolisa eine höhere Sensitivität bei HCV-Antikörper-positiven DSS-Eluaten. Aus diesem Grund wurden Proben von neu diagnostizierten HIV-Infektionen unter Verwendung des Monolisa untersucht. Des Weiteren wurde ein Aviditätstest zur Unterscheidung von frischen und bereits länger bestehenden Infektionen etabliert. Für alle Genotypen wurde ein optimaler Aviditätsindex (AI) Cut-off von 40% ermittelt, woraus ein Zeitraum von 364 Tagen für die Klassifizierung als frische HCV-Infektion folgte. Um aktive und ausgeheilte Infektionen zu unterscheiden, wurden positive Proben mittels RT-qPCR- und Western-Blot analysiert. Die Genotypisierung und Identifizierung resistenzassoziierter Substitutionen (RAS) erfolgte mittels Sequenzanalyse.

Von den insgesamt 6.097 untersuchten Proben der Jahre 2015-2017 waren 396 HCV-ELISA-reaktiv (6,5%). Von diesen wurden 256 (64,6%) als aktive und 140 (35,4%) als ausgeheilte Infektionen identifiziert. Ein hoher Anteil der HCV-Koinfektionen wurde bei intra-venösen Drogenkonsumenten (PWID, 77,8%, n=168/216), in der Altersgruppe der 30-39 Jährigen (9%, n=179/1.978) und bei Menschen osteuropäischer Herkunft (38,3%, n=124/324) beobachtet. Im Vergleich zum Jahr 2016 hat sich der Anteil der ausgeheilten Infektionen im Jahr 2017 bei der Gesamtzahl der Patienten ($p < 0,01$) sowie insbesondere bei Personen ausländischer ($p < 0,01$) und osteuropäischer ($p < 0,05$) Herkunft erhöht. Die HCV-Subtypen (St) -1a, St-3a und St-1b waren mit 33,9% (n = 79/233), 33,5% (n = 78/233) und 23,3% (n = 52/233) vorherrschend. In den Proben der Diagnosejahre 2015, 2016 und 2017 wurden RAS in insgesamt 69% (n=29/42), 60,8% (n=48/79) und 56,4% (n=22/39) Proben detektiert. Der Anteil der St-1a- und St-1b-Proben, deren HCV-Sequenz gegen DAAs resistent ist, war in allen untersuchten Jahren hoch (25% - 42,9%).

Der beobachtete Anstieg des Anteils der ausgeheilten HCV-Koinfektionen kann auf die DAA-Therapie zurückgeführt werden. Es sind jedoch weitere Anstrengungen erforderlich, damit Gruppen mit weiterhin hoher HCV-Prävalenz von dieser Behandlung profitieren. Der Nachweis von RAS in Proben von St-1a und St-1b impliziert die weitere genaue Beobachtung, um die Wirksamkeit von DAAs aufrechtzuerhalten.

1 INTRODUCTION

1.1 Hepatitis C virus

Hepatitis C virus (HCV) is the most common cause of chronic liver disease, cirrhosis, and hepatocellular carcinoma (HCC), as well as the main reason for liver transplantation worldwide. The virus was first isolated from the serum of a person with non-A, non-B hepatitis in 1989 (Choo *et al.*, 1989).

HCV is an RNA virus that is classified under the family flaviviridae (Lauer and Walker, 2001). It replicates in the cytoplasm of hepatocytes and is not directly cytopathic. Persistence of the infection depends on rapid production of virus and continuous cell-to-cell spread, together with a lack of strong T-cell immune response to HCV antigens. HCV has a high turnover rate (1,010 to 1,012 virions per day) with a half life of 2-3 hours (Neumann *et al.*, 1998). Due to its rapid replication and the lack of error proofreading ability by the RNA polymerase, the genome mutates very often (Bukh *et al.*, 1995). Currently, there are seven known genotypes (numbered 1 to 7) and more than 50 subtypes (NIHCDC, 2002). The frequent HCV mutations and the existence of various subtypes have rendered the search for an HCV vaccine difficult (Chen and Morgan, 2006).

1.2 Epidemiology, transmission and natural History

1.2.1 Global epidemiology of HCV infection

According to the estimated report of World Health Organization (WHO), there are about 71 million people living with HCV infection in the year 2015, which accounts for 1% of the population worldwide. The infection is not uniformly spread in different countries, with a prevalence ranging from 0.5% to 6.5%. The rate varies from 0.5% to 1.5% in Western countries and Australia reaching to 2.3% in South-east Asia and eastern Mediterranean regions, 3.2% in China, 0.9% in India, 2.2% in Indonesia and 6.5% in Pakistan (Petruzziello *et al.*, 2016b) (Fig. 1).

Since most acute infections are not detected clinically, it is hard to determine the rate of new HCV infections. As reported by Vogel *et al* (Vogel *et al.*, 2009), less than 25% of the acute cases are clinically presentable. Moreover, the duration of infection can not be known at the time of diagnosis. However, it is estimated that the number of new infections has shown a significant increase in the last decades (Mauss *et al.*, 2018).

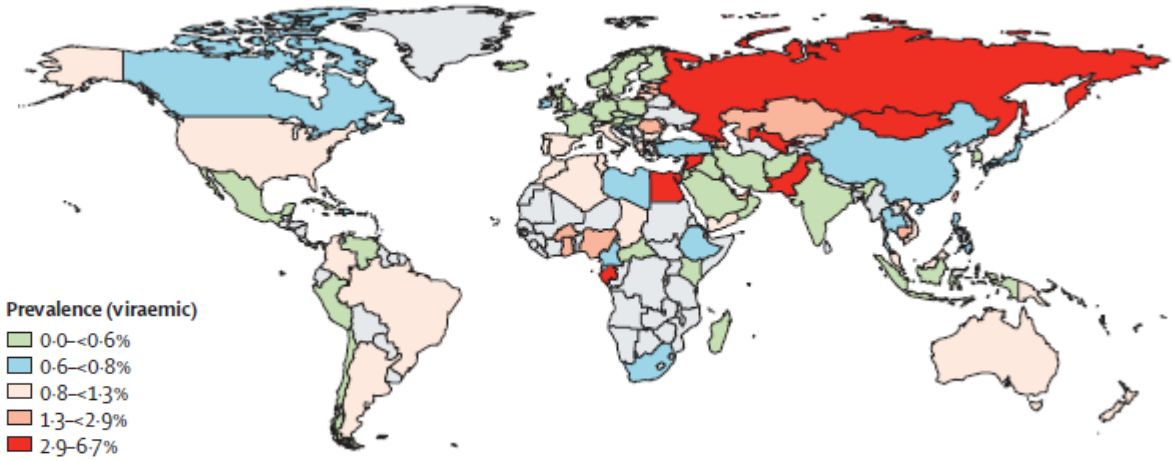


Fig. 1: Global HCV prevalence (Blach *et al.*, 2017).

1.3 Routes of transmission

1.3.1 Health-care-associated transmission

In countries where infection control measures were not enough, HCV infection can be associated with the use of unsafe injection practices and procedures like surgery, dental care, unscreened blood transfusions and renal dialysis (Candotti *et al.*, 2001, Prati, 2006, Mohsen *et al.*, 2015, Dhiman *et al.*, 2016). In the year 2010, worldwide, the use of unsafe injection devices for health care purposes was 5% (Pepin *et al.*, 2013) and these unsafe injections would lead to almost 315,000 new infections every year (Pepin *et al.*, 2014). Furthermore, the excessive use of injections (Simonsen *et al.*, 1999) together with poor injection practices can lead to increased HCV transmission (WHO, 2018).

1.3.2 Transmission among people who inject drugs

Injection drug use (IDU) is the most commonly reported cause of acute HCV infection. It has been shown that most of the new infections are acquired in individuals who are involved in the use of illegal drugs. The prevalence of anti-HCV antibodies among people who inject drugs (PWIDs) can be up to 70% with a significant variation in different regions, risk behavior, socioeconomic status etc. suggesting the efficient transmission of the virus through blood contact (Sutton *et al.*, 2008). HCV infection has also been related to the use of recreational drug use like methamphetamine in a sexual context or intranasal cocaine use, which might be because of blood on the shared straws or sniffing paraphernalia. This could partly indicate the recent increase of HCV in HIV positive men who have sex with men (MSM) (Schmidt *et al.*, 2011, Boesecke *et al.*, 2015).

1.3.3 Blood transfusion

In the past, blood transfusion or the use of blood product has been described as a major risk factor for HCV transmission. In some studies, more than 10% of the individuals who have received blood were infected (Alter *et al.*, 1989). Nevertheless, the screening of blood donations since the beginning of 1990s has drastically decreased this transmission route. In high-income countries, blood donors are at least screened for the presence of anti-HCV antibodies and HCV RNA. The transmission risk is now estimated to be between 1:500,000 and 1:1,000,000 units (Pomper *et al.*, 2003). Before the introduction of HCV screening, more than 90% of patients in cohorts of multiply transfused patients were infected with the virus (Francois *et al.*, 1993).

1.3.4 Organ transplantation

Individuals who have received organ transplants from HCV positive donors have a high risk of getting the infection. The rate of transmission has been shown to differ from 30% to 80% (Pereira *et al.*, 1991, Roth *et al.*, 1994). Therefore, most transplant organizations have devised strategies for screening and selection of organs from HCV positive donors (Mauss *et al.*, 2018).

1.3.5 Other modes of transmission

The other modes of transmission include mother-to-child transmission, which affects 4-8% and 10.8-25% of children who are born from women with HCV infection and HIV/HCV coinfection, respectively (Benova *et al.*, 2014). Sexual transmission of HCV is rare in heterosexual couples. But, the infection mostly occurs in HIV-positive individuals, especially in MSM (WHO, 2018).

1.4 Clinical manifestations of HCV infection

1.4.1 Acute hepatitis

By definition, acute hepatitis applies to the occurrence of clinical signs or symptoms of hepatitis for duration of 6 months or less after the initial exposure to HCV. In as early as 2 weeks of acute infection, HCV RNA may become detectable in the serum/plasma (Blackard *et al.*, 2008) (Fig. 2). Consistent with the course of acute hepatocellular injury, aminotransferases can become elevated in 6-12 weeks (range 1-26 weeks) and they may even be more than 10-30 times above the normal upper limit. Although the detection of antibodies might take several months, it can usually be detected at about 8 weeks after exposure.

However, the majority of the patients who have got the infection recently will be asymptomatic and have a clinically non-apparent or mild course. Symptoms of acute hepatitis include nausea, malaise and right upper quadrant pain. In patients who show these symptoms, the illness usually last for 2-12 weeks. After clinical resolution of the symptoms, the aminotransferases will normalize in about 40% of patients. HCV RNA can be lost in less than 20% of patients indicating a cured infection. Fulminant hepatic failure due to acute HCV infection can happen in patients who have chronic hepatitis B (HBV) infection (Chu *et al.*, 1999). Unless otherwise the clinical suspicion is high, only few patients will be tested for HCV RNA or antibody seroconverion. Nevertheless, in the majority of infected patients, there will be a persistance of HCV RNA and and a development of chronic state (Blackard *et al.*, 2008).

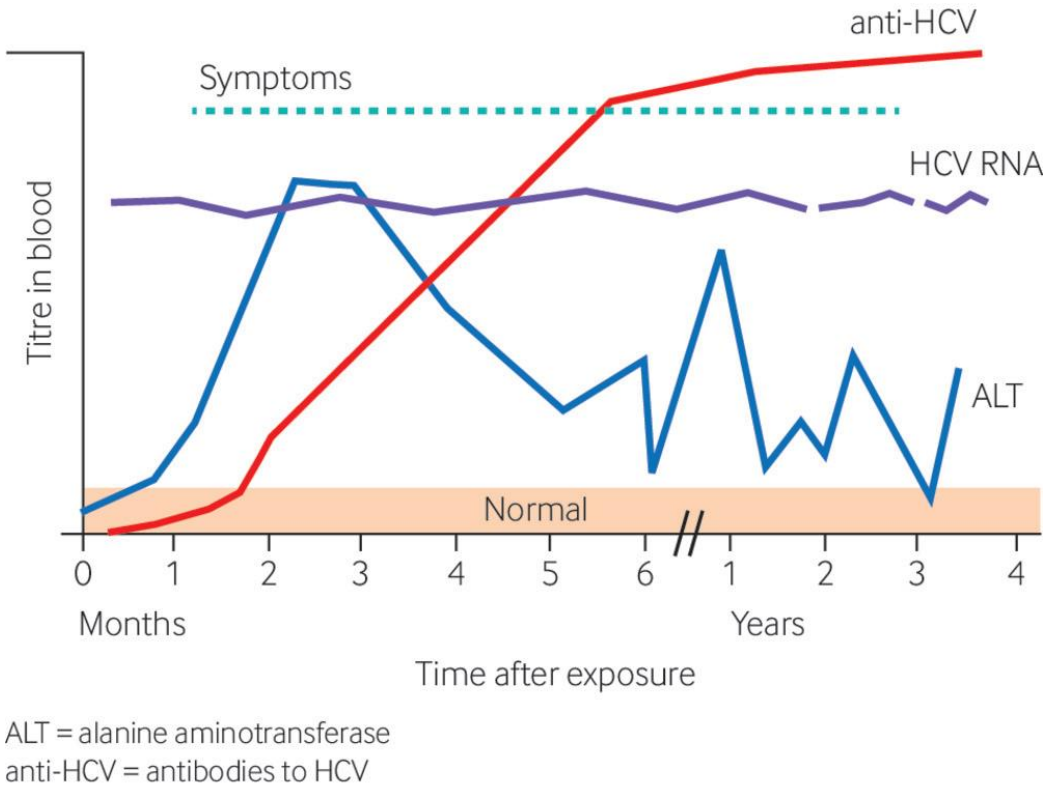


Fig. 2: Changes in the blood titers of markers for HCV infection over time (Ahmad, 2017).

1.4.2 Chronic hepatitis

In about 80% of HCV infection, the immune system is unable to completely eliminate the virus during the acute phase of infection. If the viral replication continues after six months of

infection, it is considered chronic. At this stage of the disease, most patients are asymptomatic and there might not be symptoms such as myalgia, fatigue or arthralgia. The level of aminotransferases might be moderately increased or it can also be normal (Scott and Gretch, 2007). The change in chronic infection is variable depending on factors such as higher body mass index, male gender, fatty liver, age of more than 40 years, coinfection by HIV and alcohol consumption (Thomas and Seeff, 2005). After 10 to 30 years, almost 10 to 20% of the patients develop cirrhosis. The cirrhosis may be related to liver failure, as decomposition after a portal hypertension (gastrointestinal bleeding, ascites, etc.). The risk of death due to complications of cirrhosis is 4% per year and the risk of having HCC is about 1 to 4% per year. Almost 39% of patients with HCC die within a year after its diagnosis (Thomas and Seeff, 2005, Scott and Gretch, 2007). In general, patients with cirrhosis have a five years survival rate of 50% (Morozov and Lagaye, 2018) (Fig. 3).

1.4.3 Extrahepatic manifestations

HCV infection can cause extrahepatic manifestations (Cacoub *et al.*, 2016). The most common co-morbodities associated with HCV infected persons include depression, diabetes mellitus and chronic renal disease. These morbidities in some cases are directly caused by HCV and therefore can be referred to as extrahepatic manifestations. These manifestations are likely to be affected by treatment and their prevalence is usually independent of the stage of liver fibrosis (Rutter *et al.*, 2015, Younossi *et al.*, 2016).

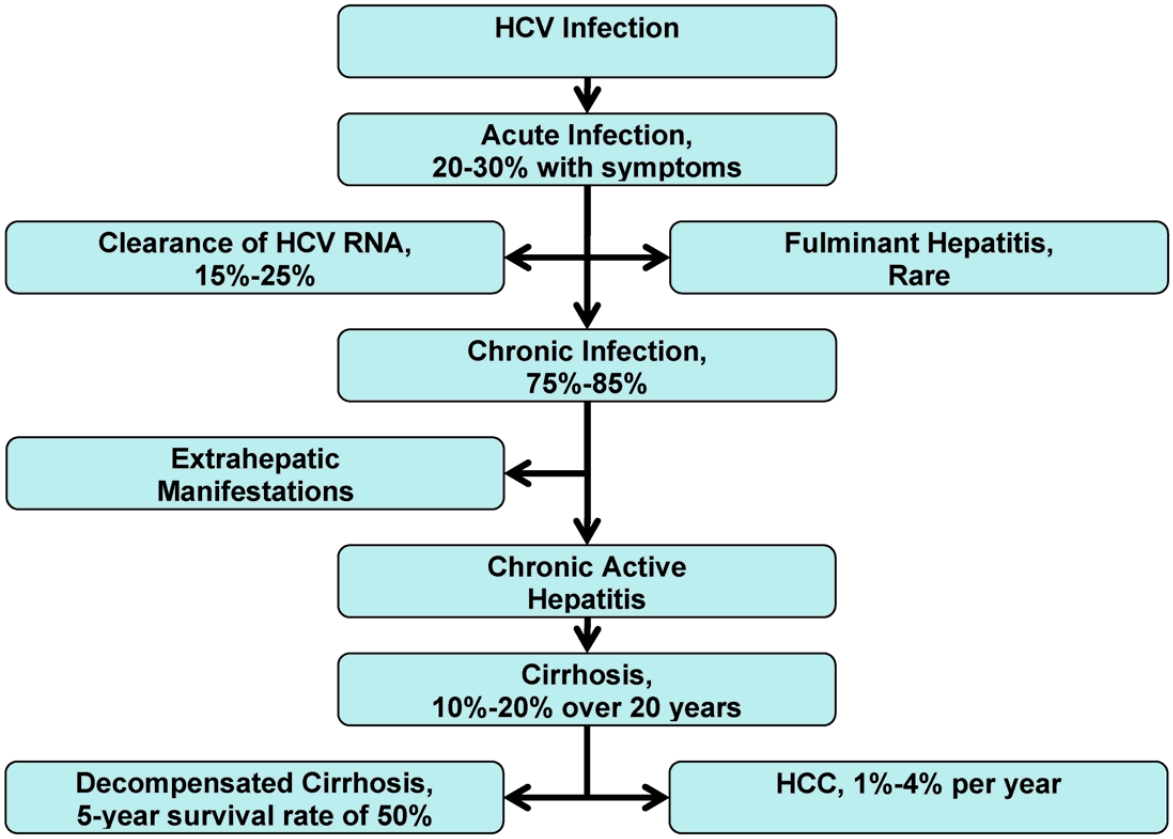


Fig. 3: Natural History of HCV Infection (Chen and Morgan, 2006).

1.5 Viral structure and genome organization

1.5.1 Viral structure

HCV is an enveloped virus (Simmonds *et al.*, 2012). It mainly replicates in the hepatocytes of infected patients (Wieland *et al.*, 2014) and the replication was found to be dependent on the liver specific microRNA-122 (miR-122) (Jopling *et al.*, 2005, Gottwein and Bukh, 2008). The nucleocapsid is formed by the viral genomic RNA associated with the capsid protein (core), which is spherical and about 30 nm in diameter (Fig. 4). The infectious virion comprises the nucleocapsid covered by a lipid-containing envelope which is derived from the host's endoplasmic reticulum (ER) membranes. The envelope has two glycoproteins E1 and E2, which are responsible for viral entry to hepatocytes through various host cell receptors (Douam *et al.*, 2015). The virus particles which were generated *in vitro* and *in vivo* have a size of 40-80nm as observed by electron microscopy (Gastaminza *et al.*, 2006, Calattini *et al.*, 2015).

1.5.2 Genome organization

HCV has a single-stranded RNA genome which is about 9.6 kilo bases in length and encodes a single and large polyprotein of almost 3,000 amino acids. The polyprotein is cleaved after translation into many structural and nonstructural (NS) peptides. The structural components include 2 envelope glycoproteins (E1 and E2) and a nucleocapsid core (C). The NS proteins are labeled NS1 through NS5 (Major and Feinstone, 1997). The specific functions of each NS proteins have not been described in detail. NS3 has both protease and helicase activities, and the NS5 region has an RNA-dependent RNA polymerase activity for viral RNA replication. The enzymatic activities can serve as potential targets for antiviral drugs. The viral genome also has highly conserved 5' and 3' untranslated regions (Major and Feinstone, 1997, Honda *et al.*, 1999, Kolykhalov *et al.*, 2000). The 5' untranslated region (UTR/NTR) has a ribosomal entry site for initiation of viral protein translation. The 3' untranslated region has structured RNA elements, which are used for the purposes of viral replication and translation (Hoofnagle, 2002) (Fig. 4).

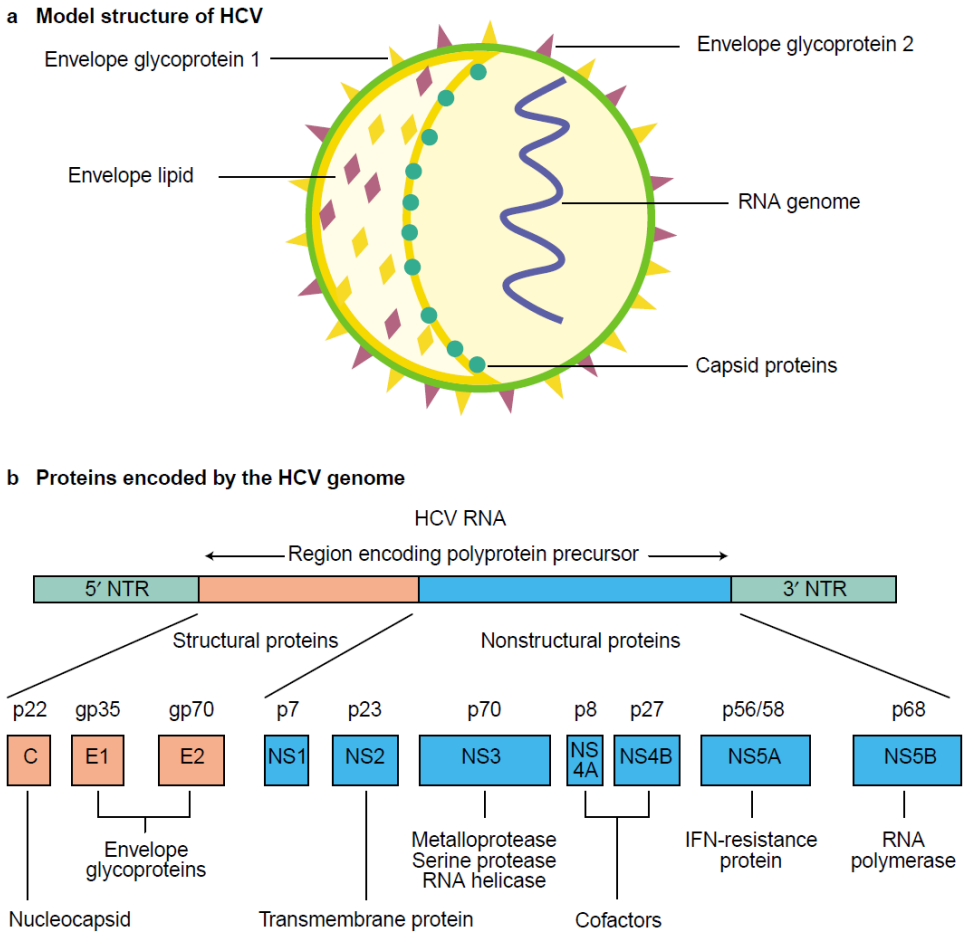


Fig. 4: HCV: Model structure and genome organization (Anzola and Burgos, 2003).

1.6 HCV Genotypes

Advances in the analysis of sequences has resulted a remarkable increase in the number of publications with open reading frame (ORF) sequenced HCV isolates (Bukh, 2016). The phylogenetic analysis carried out by Smith *et al* (Smith *et al.*, 2014) has confirmed the presence of 7 major genotypes and 67 subtypes, after which many additional subtypes have been confirmed (Li *et al.*, 2014, Lu *et al.*, 2015, Lu *et al.*, 2016, Wang *et al.*, 2019). Subtypes 1a, 1b, 2a, 2b, 2c, 3a, 4a, 4d, 5a and 6a are clearly defined worldwide or in a certain groups in the population (Simmonds, 2013). The genomes of HCV isolates grouped into major genotypes differ by about 30% at the nucleotide and amino acid level while the subtypes can typically vary by up to 10%. Therefore, HCV has a high degree of genetic heterogeneity in its whole genome with important implications for treatment, diagnosis and development of an effective vaccine. Although naturally occurring intra- or intergenotypic recombinants are rare, the 1b/2k intergenotypic recombinant has spread and gained epidemiologic importance (Kalinina *et al.*, 2002, Galli and Bukh, 2014).

1.6.1 Global distribution of HCV genotypes

The worldwide distribution of each HCV genotype differs in various geographical locations. HCV genotype 1 is the most common and has a wide geographical spread, representing 46% of all HCV infections. HCV genotype 3 is the next prevalent genotype which accounts for 30% of global infections, and it is relatively common in Australia, south Asia, and in some countries in Europe. HCV genotypes 2 and 4 account for 9-13% of HCV infections and are limited in their global distribution. HCV genotype 2 has a higher prevalence in West Africa and Asia, while the incidence of HCV genotype 4 infection is higher in North Africa, central and eastern sub-Saharan Africa and the Middle East. HCV genotypes 5, 6 and 7 are restricted in their geographical distribution, with genotype 5 common in South Africa and genotype 6 widespread in East and South-east Asia, while genotype 7 infections have been reported in very small proportions in individuals from the Democratic Republic of Congo (Gower *et al.*, 2014, Smith *et al.*, 2014) (Fig. 5).

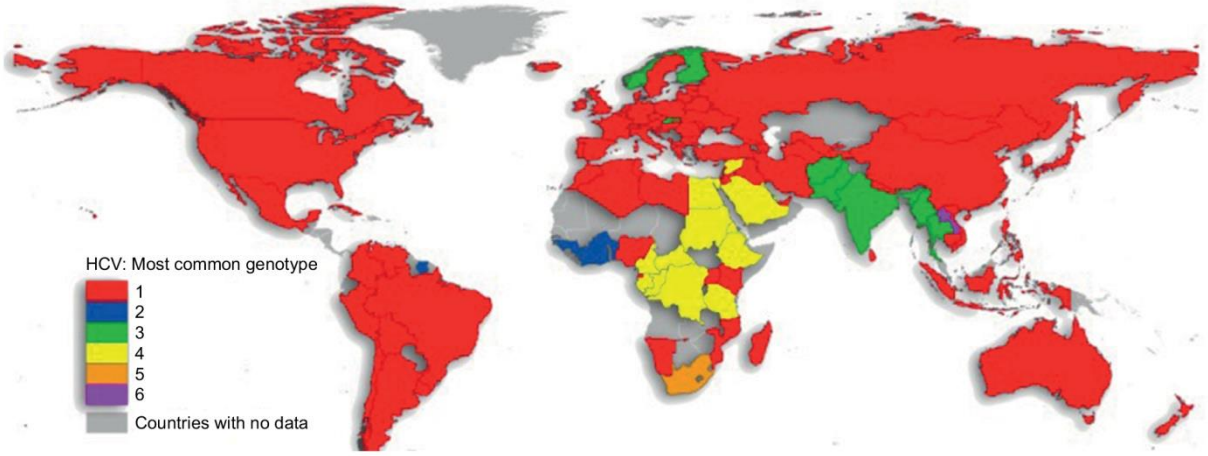


Fig. 5: Global distribution of HCV genotypes (Burstow *et al.*, 2017).

1.7 Diagnosis of HCV infection

1.7.1 Diagnosing acute HCV

1.7.1.1 Serological assays

Using sera or plasma samples HCV infections can, in principle, be diagnosed by detection of viral antigen or antibodies using enzyme immunoassays (EIAs). The first generation of HCV EIAs was able to identify seroconversion with sensitivities typically not better than 80% and window periods of infectivity between 12 to 26 weeks. Moreover, some patients never seroconverted with these assays (Cao *et al.*, 2011, Marwaha and Sachdev, 2014). By adding additional antigens the window period has been somewhat diminished to about 10 to 24 weeks in the second generation EIAs and to 9-23 weeks in the third generation EIAs. A significant improvement resulted by the development of combination assays that detect two markers of the HCV infection, antigen and antibody. These sandwich ELISAs are designated as “fourth generation” or “antigen-antibody combo tests” and reach sensitivities of 100% and specificities of 99.5% (Brandao *et al.*, 2013, Marwaha and Sachdev, 2014). With these assays the window period was further reduced by about 28 days and the combo tests considerably improved diagnosis in immunocompromised patients (Nastouli *et al.*, 2009, Yang *et al.*, 2011).

There are at least two broadly available commercial fourth generation ELISAs which can detect antigen (Ag) and antibody (Ab) simultaneously. These are the Monolisa Ultra Ag/Ab assay (Marnes-la-Coquette, France) and the Murex Ag/Ab combination assay (Abbott Murex, Abbott Park, IL) (Laperche *et al.*, 2005b, Brandao *et al.*, 2013). In a previous study the Murex

assay has been reported as more sensitive than the Monolisa assay using a panel of HCV window period samples, particularly in recognizing subtype 3a infections (Tuke *et al.*, 2008).

1.7.1.2 Molecular assays

The measurable HCV RNA serum concentrations appear within few days after infection. For HCV RNA detection, qualitative sensitive assays with a lower detection limit of less than or equal to 50 IU/ml are required, for instance transcription-mediated amplification assay (TMA), qualitative RT-PCR or the newer real-time PCR systems. However, HCV RNA may vary during the phase of acute infection, making a second HCV RNA test mandatory several weeks later in all patients with a suspicion of acute HCV who tested negative. When HCV RNA is identified in seronegative patients, acute HCV is more likely. If patients are positive for HCV RNA and anti-HCV antibodies, it might be difficult to differentiate between acute and acutely exacerbated chronic HCV. The detection of anti-HCV IgM cannot distinguish between these possibilities because of its presence in both situations. In rare cases and especially when there is a low amount of inoculum, HCV infection may only be associated with a temporal HCV RNA detection or solely by markers of the innate immune response (Heller *et al.*, 2013).

1.7.2 Diagnosing chronic HCV

Chronic HCV should be considered in patients who present morphological, clinical, or biological signs of chronic liver disease. When chronic HCV is suspected, testing for HCV antibodies by second and third generation EIAs is enough because their sensitivity is more than 99%. When anti-HCV antibodies are detected, the presence of HCV RNA has to be confirmed in order to distinguish chronic and resolved HCV infections (Mauss *et al.*, 2018).

1.7.3 HCV incidence assays

The measurement of HCV incidence, the rate of newly infected cases in a population, is an essential epidemiological tool used to identify populations at risk and to monitor the impact of public health interventions at a population level (Deltenre, 2015). However, methods to estimate the incidence of HCV are challenging, primarily because HCV infection is frequently asymptomatic (Klimashevskaya *et al.*, 2007, Gaudy-Graffin *et al.*, 2010). Moreover, it is clinically important to differentiate recently acquired HCV infections from chronic infections and to identify primary infections that are asymptomatic. The only reliable laboratory means to classify an infection as recent is to demonstrate seroconversion to anti-HCV, which is very

difficult to accomplish outside of prospective studies (Kanno and Kazuyama, 2002). However, prospective cohort studies are expensive, time-consuming, difficult to maintain, and prone to biases (Greenland, 1977, Cox *et al.*, 2009). For discrimination between recent and long standing infections HCV antibody avidity assays can be used and applied for estimating HCV incidence rates using cross-sectional research data (Patel *et al.*, 2016). It has previously been shown that HCV IgG antibody avidity, the binding capacity of maturing HCV-specific IgG antibody to antigen, increases progressively with time after exposure to an immunogen due to rapid mutations in the DNA coding for the variable part of the antibody (Inouye *et al.*, 1984). Therefore, the estimation of incidence from cross sectional surveys, using 'incidence assays' that distinguish 'recent' from 'non-recent' infections, have attracted a wide interest.

1.7.4 HCV genotyping

HCV genotyping can be done by using direct sequence analysis and reverse hybridisation technology. The first assays were designed to analyze only the 5'UTR, which causes a high rate of misclassification mostly at the subtype level. Current assays were improved by additionally analyzing the coding regions, particularly the genes which encode the non structural protein NS5B and the core protein, both of which show non-overlapping sequences between genotypes and subtypes (Bowden and Berzsenyi, 2006).

1.8 Treatment of HCV

1.8.1 Therapeutic management

For more than a decade, the treatment of choice for HCV related chronic infection was the combination of pegylated interferon alpha (peg-IFN) and ribavirin (RBV), which provided a sustained viral clearance in half of the patients infected with genotype 1, and around 75% and 60% of those infected with genotype 2 and genotype 3, respectively. Nevertheless, due to a non-satisfactory response, high severity and frequent side effects, the pharmaceutical companies were urged to develop more effective and well-tolerated drugs, termed Direct-Acting Antivirals (DAAs), which have revolutionized the treatment of HCV infection. The interferon free treatment with DAAs offers a high chance for sustained HCV elimination and prevents the progression to liver disease. These new oral DAAs provide a sustained virological response (SVR) in almost 95% of the treated patients (Coppola *et al.*, 2014, Coppola *et al.*, 2015). As of May 2018, the Food and Drug Administration (FDA) had recognized 13 DAAs from four classes (Table 1) and many fixed dose combination (FDC) DAAs for the treatment of patients with HCV infection (WHO, 2018).

Table 1: Direct-acting antivirals (DAAs) according to class (WHO, 2018).

NS3/4A (protease) inhbitors	NS5A inhibitors	NS5B polymerase inhibitor (nucleotide analogue)	NS5B polymerase inhibitor (non-nucleoside analogue)
Glecaprevir	Daclatasvir	Sofosbuvir	Dasabuvir
Voxilaprevir	Velpatasvir		
Grazoprevir	Ledipasvir		
Paritaprevir	Ombitasvir		
Simeprevir	Pibrentasvir		
	Elbasvir		

1.8.2 Summary of the currently available pangenotypic DAA combinations

DAAs are considered pangenotypic when they achieve high effectiveness in the treatment of all the main HCV genotypes (WHO, 2018). The suggested combination of pangenotypic DAAs for the management of HCV requires co-formulation with 2 and 3 second-generation DAAs. In so-called 'special populations' identified as HCV/HIV coinfection, chronic kidney disease (CKD), HCV/HBV coinfection and ineffective prior DAA regimens, these formulations have a strong antiviral potency (SVR >95%), good tolerance and decreased pill burden (Pol and Parlati, 2018).

1.8.2.1 Sofosbuvir/Velpatasvir

Sofosbuvir/Velpatasvir is a pangenotypic NS5A blocker of FDC. In clinical trials, it is associated with good effectiveness in genotype 1–6 infections, HIV/HCV coinfection, opioid replacement therapy (OST) patients and people with compensated and decompensated cirrhosis (Curry *et al.*, 2015, Feld *et al.*, 2015, Grebely *et al.*, 2016, Wyles *et al.*, 2017).

1.8.2.2 Sofosbuvir/Velpatasvir/Voxilaprevir

Sofosbuvir/Velpatasvir/Voxilaprevir is generally considered for treatment of HCV infected individuals who have previously failed the DAA regimen; however, certain high income countries also reported treatment-naïve HCV infected persons (WHO, 2018).

1.8.2.3 Glecaprevir/Pibrentasvir

Glecaprevir/Pibrentasvir is an FDC containing a pangenotypic NS3/4A protease inhibitor with a pangenotypic NS5A inhibitor. In clinical trials, Glecaprevir/Pibrentasvir has shown strong efficacy in genotype 1–6 infections, compensated cirrhosis, including in patients with renal

insufficiency and end-stage renal disease (Forns *et al.*, 2017, Gane *et al.*, 2017, Asselah *et al.*, 2018). It is contraindicated for individuals with decompensated cirrhosis (WHO, 2018).

1.8.2.4 Sofosbuvir/Daclatasvir

Clinical trials have shown strong efficacy of the combination of Daclatasvir and Sofosbuvir in genotype 1–4 infections, people with decompensated liver disease, liver transplant recipients and those with HIV/HCV coinfection (Lacombe *et al.*, 2017, Rockstroh *et al.*, 2017, Antonini *et al.*, 2018). Data also show that the combination of Sofosbuvir/Daclatasvir is also useful in genotype 5 and 6 infections (Iwamoto *et al.*, 2017).

1.8.2.5 Other DAA regimens

Additional evidence could suggest in the future that other DAA regimens (e.g. Sofosbuvir/Ravidasvir) are pangenotypic or that current pangenotypic DAA regimens may be used in more groups (e.g. children and teenagers < 18 years of age) (WHO, 2018).

1.9 HCV resistance to DAAs

The knowledge regarding the principles of how drug resistance viruses emerge is important during the use of antiviral therapies. HCV is a virus that replicates very quickly (billons of viruses/day) and the newly produced virus has an enzyme that results, on average, 1-3 errors per replication cycle. Most of these errors have no effect on viral progeny or result in a progeny that is non-replication competent (i.e. dead viruses). However, for some of the newly produced viruses, the transcription errors may result in the alteration of coding regions which, by chance, alter the susceptibility of the virus to one or more drugs used for the treating the virus. The emergence of these drug resistant viruses mostly occurs when drug levels are sub-therapeutic, which eventually create a selective pressure for the resistant viruses to come out as dominant species. The newly produced resistant viruses have a selective advantage that helps them to grow and replicate in the presence of antiviral drugs. In some patients with chronic HCV infection, viral variants which contain substitutions associated with resistance to the HCV DAAs can be detected before the initiation of antiviral therapy. In the case of NS5A inhibitor containing regimens, this in particular may negatively affect treatment response. These kinds of substitutions are often called as baseline resistance associated substitutions (RAS) (AASLD, 2018).

Resistance viruses can also be selected and enriched in patients with DAA regimen failure. The substitutions that are contained in these viruses are designated as treatment emergent or treatment selected RASs. The RASs in NS3 and NS5A are frequently selected in patients who have a failure of NS3 and NS5A containing regimens, respectively. On the other hand, the selection of NS5B nucleotide RASs is rare (1% of failures) even after the exposure to a failing DAA regimen which contains a nucleotide inhibitor (Svarovskaia *et al.*, 2014). This rare selection of RASs is due to the highly conserved catalytic site where the nucleotides bind, making the substitutions in this region very rare which is mostly referred to as a high barrier to resistance. Moreover, any of such substitution would likely cause the virus to become replication-incompetent. However, NS5A RASs can maintain the high viral replication competence (also called relative fitness) in the absence of continued drug pressure, which allows them to be the dominant viral quasispecies for many years in comparison to the NS3 protease or NS5B nucleotide polymerase inhibitor RASs, which are relatively less fit and can disappear from peripheral blood within a few weeks to months, being dominated by the more fit and wild type virus species (AASLD, 2018).

In general, drug specific RASs need to be present in at least 15% of the viruses of a given patient to reduce the likelihood of achieving SVR (Pawlotsky, 2016). Drug specific RASs that are found at a lower frequency may not convey sufficient resistance to lower SVR with currently available DAA regimens (AASLD, 2018).

1.9.1 Therapeutic outcomes of DAAs against various HCV genotypes

1.9.2 HCV-RAS in different genotypes

The new DAA regimens achieve higher SVR rates in all genotypes, but the therapeutic efficacy varies at the level of genotypes as well as subtypes and even in more difficult to treat specific populations (e.g., HCV subtype 1a, genotype 3 and genotype 4 patients with compensated and decompensated cirrhosis, HCV/HIV coinfected patients, severe liver impairment patients and chronic kidney disease). DAA regimens individually, in combination (e.g. Sovaldi ®, Daklinza ®, Olysio ® with or without ribavirin) or as a fixed dose formulation (Epclusa ®, Harvoni ®, Zepatier ®, Viekira Pak ®, Vosevi ®, Mavyret ®) achieve higher SVR rates (> 95%) in treated patients with genotypes 1, 2, 5 or 6 infections. However, genotype 3 patients had SVR levels of ≤90–95%. Likewise, the viral rebound, virological breakthrough and the discontinuation of therapy were also common in cirrhotic patients (Shahid *et al.*, 2018b).

It has also been shown that single or dual DAA regimens have not been able to achieve higher SVR rates in HCV genotype 3 patients and the addition of other DAAs (i.e. triple DAA regimens) is of importance to achieve higher SVR rates for this particular genotype. In clinical studies of approved regimens, HCV genotype 4 patients with or without cirrhosis also achieved compromised SVR rates (≤85–95%) (Shahid *et al.*, 2018a). For this reason, the recently approved regimens are carefully recommended in compensated and decompensated cirrhotic patients. The mechanisms behind the variable reponses to all oral DAAs to various genotypes are not fully understood. However, high viral load, viral genome heterogeneity, disease progression and, in particular, the development of viral escape mutants are regarded as predisposing factors in this prospect (Buti and Esteban, 2016, Shahid *et al.*, 2018a).

1.9.3 Viral resistance substitutions against first-generation DAAs

Patients who take Boceprevir or Telaprevir as monotherapy can develop antiviral resistance within a few days of treatment. In all HCV infected individuals, minor resistance populations to these drugs occur at baseline and are rapidly selected with Telaprevir or Boceprevir monotherapy (Jacobson *et al.*, 2012). Likewise, prominent drug-drug interactions with many HIV antiretrovirals and calcineurin inhibitors also limit the clinical efficacy of Telaprevir and Boceprevir monotherapy (due to rapid emergence of RASs, severe drug adverse events and numerous possible drug-drug interactions, the first generation of potease inhibitors (PIs) have been suspended by the FDA to treat HCV patients in many parts of the world) (Susser *et al.*, 2011, Jacobson *et al.*, 2012). R155 is the most important position in NS3/4A serine protease (a protein implicated in HCV translation and also a possible drug active site for the design and development of PIs) where various mutations can present resistance to almost all PIs (with the exception of MK-5172) (Romano *et al.*, 2012). *In vivo* mutations at four locations (V36A/M/L, R155K/M/S/T, A156S/T and T54A) and one *in vitro* mutation (A156) have been observed and described against Telaprevir. Such mutations either individually (T54A, V36A/M, A156S, R155K/T,) or as double mutations (V36M + R155K, V36M + 156T, A156T/V) confer a low to high resistance to Telaprevir by modifying the active sites of NS3/4A serine protease (Welsch *et al.*, 2008). The pattern of resistance to Telaprevir often differs considerably among different HCV subtypes. Clinical studies have shown that antiviral resistance is much more common in patients infected with subtype 1a than subtype 1b either using Telaprevir alone or together with PEG-IFN-α plus RBV (Susser *et al.*, 2011). This is attributed to a single nucleotide polymorphism at position 155 in NS3/4A serine protease, where codon AGA encodes R in subtype 1a versus 1b (where codon CGA also encodes R)

(Welsch *et al.*, 2008, Susser *et al.*, 2011). Only a single nucleotide substitution is needed for HCV subtype 1a isolates to switch R to K at position 155, whereas 2 nucleotide changes are necessary for subtype 1b (Kliemann *et al.*, 2016). Studies have also shown that subtype 1a has a higher fitness advantage than subtype 1b isolates, which is a predisposing factor in the production of viral escape mutants and viral breakthroughs to other sites within the NS3/4A catalytic subunit and other genomic regions of 1a isolates (Welsch *et al.*, 2008, Susser *et al.*, 2011).

1.9.4 RAS against second-generation DAAs

Q80R/K polymorphism is responsible for low level resistance to Simeprevir. Clinical studies have predicted the existence of Q80K variants up to 50% in subtype 1a infected patients (approximately 20% in Europe and 50% in the US) and roughly 1% in subtype 1b isolates (Lenz *et al.*, 2012). Lower SVR rates and gradual viral decline have been documented in subtype 1a patients treated with Simeprevir based triple therapy in Phase III clinical studies (20% lower in subtype 1a than 1b) (Manns *et al.*, 2014). Q80K polymorphism and NS3 genotype screening prior to therapy is highly recommended for patients with subtype 1a to prevent any adverse events, low virological response and discontinuation of treatment during therapy. The viral variants associated with NS3 PIs can detect a reverse transcription reaction by first synthesizing cDNA, followed by a PCR and a sequencing reaction. In the US, Quest Diagnostics ® and LabCorp ® developed Q80K polymorphism and viral variant screening against NS3 PIs (Lenz *et al.*, 2012, Manns *et al.*, 2014).

1.9.5 Detection of resistance associated substitutions (RAS)

The methods used for the detection of RAS include population sequencing (Sanger sequencing) and deep sequencing (next generation sequencing [NGS]). Both methods rely on sequencing of the viral RNA, assessing the amino acid sequence, and then inferring the existence of RASs. The sensitivity of the methods differ in detecting RASs. For the purposes of clinical care and making decisions with regards to which directly acting antiviral (DAA) regimen to use, these methods can be considered similar if a ≥15% cut-off is used for determining RAS by NGS. Studies have shown that NGS at a 1% level of sensitivity can result in the detection of additional RASs that are not related to clinical failure (Sarrazin *et al.*, 2016, Zeuzem *et al.*, 2017).

However, only few reference laboratories have made these tests available in the United States and Europe, and the performance of these assays has not been externally evaluated. In-house assays are available in some local laboratories, but their reliability and performance vary. The drug resistance testing for HCV is not trivial as it requires polymerase chain reaction (PCR) amplification of the three genes encoding the NS3 protease, NS5A and NS5B proteins. This can even be problematic especially for genotypes other than 1 and 4. Due to variation in the sensitivity of population sequencing, it can be difficult to interpret the results among laboratories, whereas the cutoffs for deep sequencing are not standardized. Not all RASs are clinically relevant and their effects relay on the proportion of viral quasispecies and the existence of other RASs in the same or other regions of the genome (Pawlotsky, 2016).

1.10 HCV/HIV Coinfection

1.10.1 Epidemiology of HIV/ HCV coinfection

It is estimated that 4–5 million (11–14%) of the 35 million HIV-infected individuals worldwide are coinfected with HCV (Alter, 2006). Because HCV is transmitted mainly via blood-to-blood contact, the likelihood of coinfection depends on how HIV is transmitted. At the time of HIV diagnosis, most patients are already diagnosed with HCV. In Northern Europe, Canada and other regions where MSM is the predominant transmission group for HIV and preventive strategies for HIV and HCV are common for PWIDs, the prevalence of HCV coinfection is relatively low (5-20%), whereas in Eastern Europe, were PWIDs are the main driver of the HIV epidemic, the coinfection rates can be higher than 50% (Peters *et al.*, 2014) (Fig. 6). Although acute HCV outbreaks between HIV positive MSM have received considerable attention in recent years, the prevalence of HCV coinfection in this population remains relatively low. In EuroSIDA, a large pan-European longitudinal study that follows more than 13,000 patients with documented anti-HCV status infected with HIV, only 5% of MSM were coinfected with HCV. By contrast, 13% and 90% of people who describe heterosexual contact or IDU as their main risk of transmission of HIV are coinfected with HIV/HCV, respectively (Rockstroh *et al.*, 2013). Because heterosexual transmission of HCV is rarely documented (Terrault *et al.*, 2013), much of the high prevalence of coinfection in heterosexuals is likely explained by unreported transmission of IDU and iatrogenic HCV in immigrants (Peters and Klein, 2015).

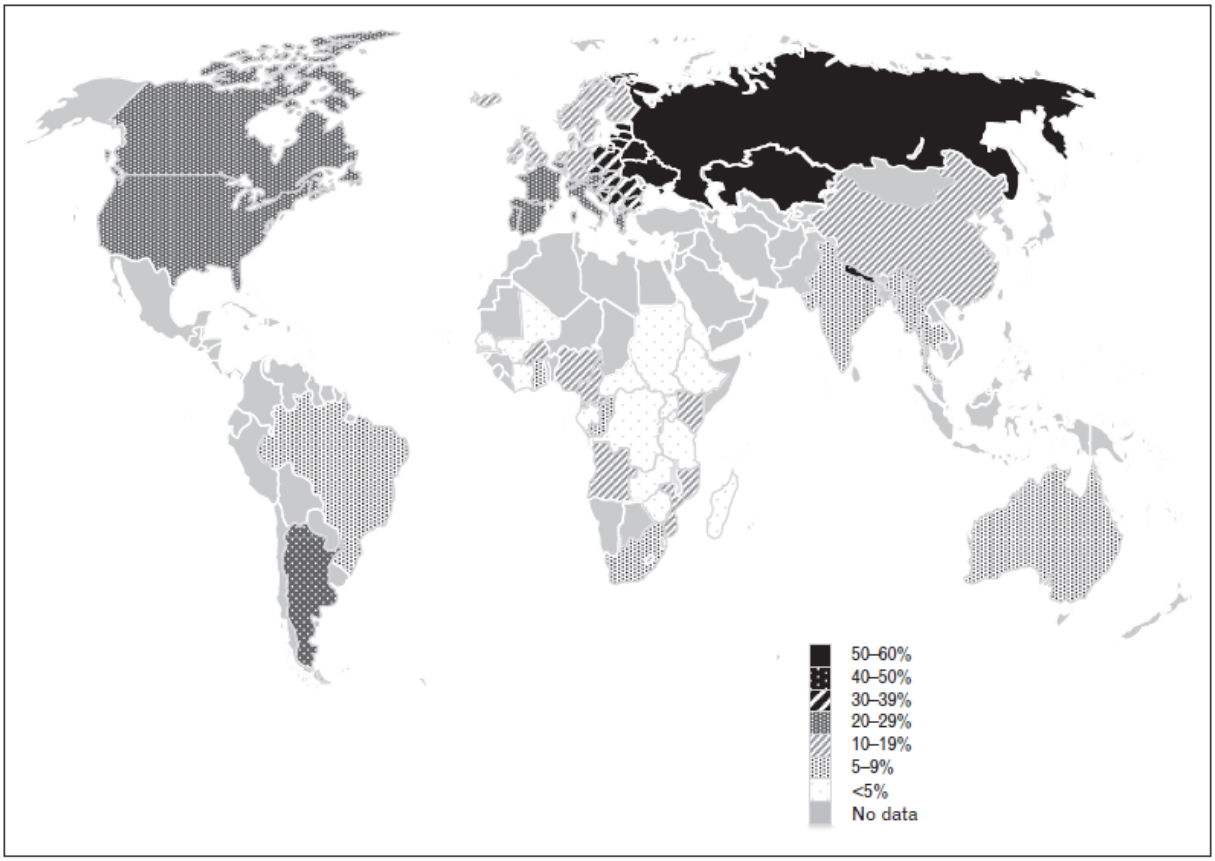


Fig. 6: Prevalence of hepatitis C virus coinfection among HIV-infected people in individual countries (Peters and Klein, 2015).

1.10.2 The situation in Germany

A population based study in Germany found a 0.3% HCV antibody prevalence in the general population between the years 2008 to 2011. The actual prevalence of HCV antibodies may be higher as this study excluded hospitalized patients, psychiatric patients and prisoners. Also, there was an underrepresentation of other risk groups such as PWIDs and other populations at higher risk of HCV infection (Poethko-Muller *et al.*, 2013). The study by Jansen *et al* (Jansen *et al.*, 2015) was also Germany's first extensive screening of plasma samples from HIV 1-positive MSM with documented date of HIV 1-seroconversion for HBV, HCV, and syphilis coinfection. In this study, 8.2% of the MSM were positive for HCV infection. Among these, 10.5% were tested HCV positive at the time point of HIV seroconversion and 48.7% had HCV viremic test after HIV seroconversion. Genotype 1 was the most commonly found (71.6%), followed by genotypes 4 (19.2%), 3 (6.8%), and 2 (2.7%). As in other Western European countries, in Germany HCV is hyperendemic in PWIDs, and regional studies have found a prevalence of 50–80% in this transmission group (Stark *et al.*, 1995, Stark *et al.*, 1997, Roy *et al.*, 2002, Backmund *et al.*, 2003).

1.10.3 Natural history of HIV/HCV coinfection

HIV coinfection adversely affects the course of infection with HCV. Coinfected individuals, especially those with advanced immunodeficiency (CD4 count <200 cells/mm^3), have substantially increased progression to cirrhosis, decompensated cirrhosis and HCC compared with HCV-monoinfected individuals (Reiberger *et al.*, 2010). In high-income countries, HCV associated liver disease has become the leading cause of death in HIV positive people, which accounts for nearly half (47%) of all deaths in the United States (Weber *et al.*, 2006). It is not clear whether HCV infection fastens the progression of HIV disease, but compared to those with monoinfection, CD4 recovery in the coinfected individuals is impaired after initiation of antiretroviral therapy (ART) (Tsiara *et al.*, 2013). In some studies, HIV/HCV coinfected individuals have shown a more rapid progression of HIV infection in relation to those who were HIV-infected alone (Rockstroh *et al.*, 2005). Assessment of the effect of HCV infection on the progression of HIV infection may be confounded by the negative health consequences of IDU, which is strongly associated with HCV infection (May *et al.*, 2015a). In persons with HIV coinfection, HCC tends to occur in people with HIV coinfection at a younger age and in a shorter period of time (Brau *et al.*, 2007).

1.10.4 Diagnosing HCV in HIV coinfection

In rare cases of HCV/HIV coinfection, the detection of HCV antibodies can be lost during an advanced immune deficiency and does not necessarily indicate viral clearance (Cribier *et al.*, 1995). Therefore, a single negative HCV ELISA antibody does not necessarily exclude exposure to HCV in HIV-positive patients, particularly in severe immune deficiency. In addition, an increase in liver transaminases (especially Alanine aminotransferase, ALT) may be more sensitive for the detection of acute HCV in HIV positive patients than repeated tests for HCV antibodies (Thomson *et al.*, 2009). However, in cases of suspected early acute HCV, the diagnosis based on HCV RNA is necessary.

HCV RNAs are found in higher concentrations in HIV positive patients than HIV negative patients with HCV monoinfection (Perez-Olmeda *et al.*, 2002). The levels of HCV viraemia can increase 8 times faster in HIV positive than HIV negative patients. The highest concentrations of HCV viremia have been reported in patients who have developed liver failure (Mauss *et al.*, 2018).

Spontaneous clearance of HCV RNA appears to be less frequent in HCV/HIV coinfection settings (Thomson *et al.*, 2009). Nonetheless, spontaneous clearance of HCV RNA has been observed in some patients with chronic HCV/HIV coinfection who have experienced a significant immune reconstitution after ART initiation, especially in patients with a favorable IL28B CC genotype (Fialaire *et al.*, 1999, Thomson *et al.*, 2009, Stenkvist *et al.*, 2014).

1.10.5 Treatment of HCV in HIV coinfection

The recommendation for the treatment of HIV coinfected patients is the same way as moninfected patients. Several studies have shown that effective treatment of coinfection can significantly reduce the risks of preexisting liver disease in HIV-positive patients (Mira *et al.*, 2013). This means that once virus clearance is reached, the prognosis of liver disease improves dramatically (even in the existence of an already developed liver cirrhosis) and once HCV is eradicated, further liver complications are highly unlikely for patients with low-grade liver fibrosis. Therefore, irrespective of the stage of liver fibrosis, treatment for HCV should be considered for all patients who are living with HCV/HIV coinfection (Mauss *et al.*, 2018).

2 AIMS OF THE STUDY

The advent highly potent and safe DAAs against HCV and changes in the guidelines for HIV treatment that recommend starting therapy as soon as possible could lead to a dramatic change in the epidemiology of both infections, particularly in high risk groups. Therefore, ensuring effective prevention and evidence-based health policy decisions in times of such paradigm shifts surveillance using the best possible epidemiological and molecular methods is mandatory.

In high-income countries, the burden of HCV is high among PWIDs and increasing among HIV-infected MSM, who are the key populations for HCV transmission (Martin *et al.*, 2015). HCV treatment for these populations could become an important prevention strategy, especially in the IFN-free DAA era. Hence, there is a need to explore whether the treatment can reduce HCV prevalence and prevent transmission among these transmission groups. Germany has been one of the most popular destination and host countries for asylum seekers in Europe during recent years with the vast majority of asylum seekers arriving between July 2015 and February 2016 (Grote, 2018). The immigration of HCV and HIV-infected persons coming from countries which are traditionally characterized by a high endemicity rate can be a most important factor contributing to the viraemic pool in the country (Negro, 2014). However, a representative data on the status of HIV/HCV coinfection in the country is so far not available.

While elimination of HCV infection in Germany seems to be feasible and cost-effective with the new pangenotypic interferon-free DAAs (Krauth *et al.*, 2019), there is still a need to genetically characterize the currently circulating HCV strains to guide effective initial therapies strategies and to ensure the specificity and accuracy of viral diagnostics (AASLD, 2018, EASL, 2018).

Therefore, this study aims to conduct an up-to-date epidemiological and molecular surveillance of HCV coinfections among new HIV infections diagnosed between the years 2015 and 2017 in Germany. The study also presents data on the prevalence and incidence of HCV, on circulating HCV genotypes/subtypes, on existing resistances in relation to the main transmission groups, sex groups and origin of the infected individuals. This knowledge enables to have an early recognition of trends, implementation of targeted and appropriate prevention measures or therapy optimization and the making of evidence-based health policy decisions in the country.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipments

Equipment	Model	Company
Incubator	Heraeus B 6030	Heraeus, Germany
RNA extractor	NucliSens easyMAG	BioMérieux, France
Gel documentation	BioDocAnalyze	Biometra, Germany
Gelelectrophoresis-Combs	Mini Elektrophorese System	VWR International,Germany
Laminar	Bleymehl-Reinraumtechnik	Inden-Pier, Germany
Microwave		Bosch, Germany
Thermocycler	T 300 Thermocycler	Biometra, Germany
PCR Cycler	Biometra Trio	Biometra, Germany
Vortexer	Top Mix	Thermo Scientific, USA
Centrifuge	Rotilabo®-Mini-Zentrifuge	Carl Roth, Germany
ELISA plate washer	Infinite F200 Microplate	Tecan, Switzerland
ELISA plate reader	HydroFlex™ Microplate	Tecan, Switzerland
Real-Time PCR Cycler	LightCycler 480 II	Roche, Switzerland

3.1.2 Chemical reagents

Reagent	Company
100bp DNA Ladder (LS100)	Invitrogen, USA
6x Loading buffer bromophenol blue	NEB, England
Tris-EDTA Buffer	Promega, USA
1xTAE Buffer	Thermo Scientific, USA
Agarose Standard Rotigarose	Carl Roth, Germany
dNTP Mix	Thermo Scientific, USA
GelRed	Biotium, USA
NucliSense easyMAG Magnetic Silica (Beads)	BioMérieux, France
NucliSENS easyMAG Extraction buffer 1	BioMérieux, France
NucliSENS easyMAG Extraction buffer 2	BioMérieux, France
NucliSENS easyMAG Extraction buffer 3	BioMérieux, France
NucliSENS easyMAG Lysisbuffer	BioMérieux, France

3.1.3 Kits

Kit	Company
HotstarTaq Master Mix	Qiagen, Netherlands
Monolisa™ HCV Ag-Ab ULTRA V2	Bio-Rad, USA
Monolisa anti-HCV PLUS Version 3	Bio-Rad, USA
*recom*Line HCV IgG	Mikrogen, Germany
RNasin	Promega, USA
SuperScript™ II Reverse Transcriptase (RT)	Thermo Scientific, USA
SuperScript™ III Platinum™ One-Step qRT-PCR	Thermo Scientific, USA
Agencourt® AMPure XP	Beckman Coulter, USA
Quant-iT™ PicoGreen™ dsDNA Assay	Thermo Scientific, USA

3.1.4 Probe

Probe	Direction	Sequence	Company
HCV-TM-5	Sense	FAM 5′CCT TGT GGT ACT GCC TGA 3′MGB	Thermo Scientific, USA

3.1.5 Filter Card

Filter Card	Company
Whatman 903 filter card	GE Healthcare Bio-Sciences Corp, USA

3.1.6 Device software

Software	Producer
BioDocAnalyze	Biometra, Germany
Geneious 11.1.5	Biomatters, Newzeland
Magellan	Tecan, Switzerland
HCV Phylogenetic Typing Tool 2.4	GenomeDetective,Belgium
NCBI Genotyping	NCBI, USA
GraphPad Prism 8.0	GraphPad Software, USA

3.1.7 Equipments

Equipment	Model	Company
96-Well-Plate	LightCycler 480 Multiwell Plate 96	Roche, Switzerland
Aspirator	FTA-1	Biosan, Latvia
Beaker	200ml	Duran, Germany
Combitip	Combitips advanced®25ml, 50ml	Eppendorf, Germany
Extraction disposable container	NucliSENSeasyMAGDisposables	BioMérieux, France
Falcon tube	15ml, 50ml	Sarstedt, Germany
Sealing foil for qPCR	LightCycler 480 Sealing Foil	Roche Molecular Systems
Gloves	NeoTouch	Ansell, Australia
Measuring cylinder	400mL, 1000ml	Brand, Germany
Multipette plus	1µl-10ml	Eppendorf, Germany
Pipettes	10, 20, 100, 200, 1000µl	Eppendorf, Germany
Pipettes, ArtTips	10, 20, 100, 200, 1000µl	Thermo Scientific, USA
Pipettes, ClipTip	10µl, 30-300µl	Thermo Scientific, USA
Pipette, automatic	e1200	Sartorius, Germany
Pipette aid	pipetus	Hirschmann, Germany
Pipette tips	10, 20, 100, 200, 1000 µl	Thermo Scientific, USA
Pipette tips, epT.I.P.S.	20-200µl	Eppendorf, Germany
Pipette tips, ClipTip	300µl	Thermo Scientitic, USA
PCR tubes	0,2ml, 1,5ml, 2ml	Sarstedt, Germany

3.1.8 Primers for 5'UTR region

Primer	Direction	Sequence	Region
235	Antisense	AGT ACC ACA AGG CCT	5´UTR
238	Sense	GAG GAA CTA CTG TCT TCA CG	5´UTR
239	Antisense	TCG CAA GCA CCC TAT CAG	5´UTR
240	Sense	CGT CTA GCC ATG GCG TTA G	5´UTR
255	Sense	AGY GTT GGG TYG CGA AAG	5´UTR
256	Antisense	CAC TCG CAA GCR CCC T	5´UTR

3.1.9 Primers for NS3 region

Primer	Direction	Sequence	Region
261	Sense	GAT CTG GCC GTG GCT GTA GAG	NS3
262	Antisense	AGC ACC TTG TAG CCC TGA GC	NS3
263	Sense	GCG GTG ACA TCA TCA ACG GCT	NS3
264	Antisense	GGG ACC TCA TGG TTG TCT CTA GG	NS3
265	Sense	CTT GCG GTG GCA GTT GAG C	NS3
266	Antisense	CCG TGG TGA TGG TCC TTA CCC	NS3
267	Sense	GCT GCA TCA TCA CCA GCC TCA C	NS3
268	Antisense	GGC ACC TTA GTG CTC TTG CC	NS3
280	Sense	GGA GGT TGC TGG CGC CCA T	NS3
281	Antisense	GCC GGG ACC TTG GTG CTC TT	NS3
283	Antisense	GCG TGT AGA TGG GCC ACT TG	NS3

3.1.10 Primers for NS5A region

Primer	Direction	Sequence	Region
284	Sense	CAT ACT CAG CAG CCT CAC TGT	NS5A
285	Sense	ACC AGT GGA TAA GCT CGG AGT G	NS5A
286	Antisense	CCA TAT AAC AGC AGA GGC GGC	NS5A
289	Sense	GCA CTA TGT GCC TGA GAG CG	NS5A
291	Antisense	CAG TGC TCA CTT CCA TGC TCA	NS5A
292	Sense	GCT GAG TTC TCT AAC TGT CAC AAG	NS5A
293	Sense	ACT GTC ACA AGT CTG CTC CGG	NS5A
294	Antisense	TCC ATC AGA GGC AAG CTC ATC	NS5A
295	Antisense	GCT GAC CTC GAT GTT GAG AGA CC	NS5A
308	Sense	ATG AAC CGG CTG ATA GCG TTC	NS5A
309	Antisense	GCTCACTTCCATGCTYACCGA	NS5A
335	Sense	AAA CCA TGT TGC CCC TAC CC	NS5A
336	Antisense	GTC TGT CAA CAT GGA GGC CA	NS5A
337	Antisense	GAG AGC CGA CCA CAA AGG AA	NS5A
362	Sense	RCY ATY GCY TCC CTC ATG	NS5A
363	Sense	AAG ATC ATG AGY GGY GAG A	NS5A
364	Antisense	GRC AGG GRC ACT TGA BRT T	NS5A

3.1.11 Primers for NS5B region

Primer	Direction	Sequence	Region
257	Sense	GGS TTY TCN TAT GAY ACC MGV TGY TTT GA	NS5B
259	Sense	GCT GYT TTG AYT CAA CNG TCA C	NS5B
270	Antisense	TAY CTG GTC ATA GCY TCC GTG AAR G	NS5B
271	Sense	ACC ACA TCM RST CCG TGT GG	NS5B
272	Sense	TCC GTG TGG RAR GAC YTS CTR GA	NS5B
275	Antisense	CTS GTC ATA GCY TCC GTG AA	NS5B
296	Sense	AGG CTA TGA CCA GGT ACT CCG	NS5B
298	Antisense	CTA CTC CTG CTT GCT GCA GGG	NS5B
299	Antisense	TTT GCC TAC TCC TGC TTG CTG	NS5B
301	Sense	CGG AGG CTA TGA CTA GGT ACT C	NS5B
302	Antisense	GGA GCA GGT AGA TGC CTA CC	NS5B
303	Antisense	TCA TCG GTT GGG GAG CAG GTA	NS5B
305	Sense	CTC CGT MTG GGA GGA CTT GC	NS5B
311	Sense	GCGWGCCTGAGAGCCTTCAC	NS5B

3.1.12 Web tool

Software	Producer
Geno2Pheno	Max Planck Institute for Informatics, Germany

3.2 METHODS (PART I)

3.2.1 Longitudinal HCV panels

For the evaluation of Murex and Monolisa Ag/Ab HCV combination assays commercially available HCV seroconversion panels from SeraCare Life Sciences, Inc. (Milford, MA, USA) were used. The panels include serial bleeds collected from two plasma donors during a period of HCV antibody seroconversion. The first bleeds were RNA positive and antibody negative. These samples had a higher HCV RNA load (>100,000 IU/ml) and can therefore be considered antigen positive based on data reported earlier (Soliman *et al.*, 2015, Thong *et al.*, 2015), while bleeds of later time points were designated as RNA and antibody positive. The panels PHV917 (M) and PHV920 (M) comprised HCV subtypes 1a and 2b with bleed durations of 152 and 35 days, respectively. This information is also provided as part of figure 1A and B. In addition to these seroconversion panels, panels of longitudinal HCV RNA and anti-HCV antibody positive serum samples were used, which contained subtypes 1b (male, 48 years old and HIV positive; n=4), 3a (male, 49 years old and HIV positive; n=3), and 4d (male, 35 years old and HIV positive; n=3) each with durations since first bleed of 168, 182, and 176 days, respectively. These were anonymous residual samples provided by a diagnostic laboratory in Berlin (Medizinisches Infektiologiezentrum Berlin) along with HCV RNA and anti-HCV antibody test results.

3.2.2 Preparation of dried serum/plasma spots

Specimens from the panels were prepared as DS/PS, by dropping the plasma or serum (100 µl) on Whatman 903 filter cards, air drying overnight at room temperature, packaging in a zip-lock bag containing desiccant packets and storing at -20°C. For use in the EIAs, antibodies were eluted from spots in 500 µl elution buffer (500 ml PBS, 0.05% Tween, 3% FCS) resulting in a 1:5 dilution.

The sensitivity of Murex and Monolisa Ag/Ab HCV combination assays was first tested by using undiluted longitudinal HCV antibody and/or antigen-positive samples from different time points (HCV seroconversion panels). Since the extent of anti-HCV antibody elution and the performance of the two assays for different antibody concentrations are not known, the DS/PS eluates (1:5 dilution) were further diluted in different amounts of an elution buffer resulting in dilutions of 1:10, 1:15, 1:50, and 1:100. The DS/PS dilutions were then compared to the undiluted samples to analyze the sensitivity of undiluted and DS/PS samples.

To further analyze the performance of both combination assays, clinical DSS eluates, which represent anonymous residual serum spots of new HIV diagnoses (for details see (Hauser *et al.*, 2017) were used for screening of potential HCV coinfections and calculation of the percentage of correct positive test results. For lack of a "gold standard" commercial assay, ELISA-reactive samples were confirmed by an in-house quantitative RT-qPCR assay, which is optimized for quantifying HCV RNA in DSS (Wang *et al.*, 2019). HCV RNA negative ELISA reactive samples (presumed resolved infections) were analyzed by Western blot to confirm seroconversion.

3.2.3 Assays for the combined Ag/Ab detection

3.2.3.1 Murex Ag/Ab combination assay

The wells of the Murex Ag/Ab HCV combination assay are coated with anti–core monoclonal antibody for the detection of HCV antigen and recombinant antigen, peptides representing the immunodominant regions of the NS3 protein and core viral antigens for the detection of HCV antibodies. The assay was performed as recommended by the manufacturer. Briefly, 50 µl of sample diluents and the same volume of specimens or controls were added to each of the microplate wells. The plates were then incubated at 37 ^{0}C for 1 h followed by five washing steps, after which 120 µl of conjugate (containing HCV antigens and a monoclonal antibody conjugated to horseradish peroxidase) was added to all wells. The microplates were then incubated for 1 h at room temperature. The microplate was washed again, 80 µl of substrate was added to each of the wells, and incubated for 30 minutes at 37 ^{0}C. Lastly, stop solution was added and the plates were read at 450/620 nm using an Infinite 200 PRO Microplate reader (Tecan). The cut-off value was determined by adding 0.2 to the mean OD of the negative control.

3.2.3.2 Monolisa Ag/Ab ULTRA assay

The test process for the Monolisa HCV Ag/Ab ULTRA was also performed following the instructions of the manufacturer. The wells of the microplates are coated with monoclonal antibodies specific for HCV capsid, NS3 and NS4 purified recombinant proteins, and a peptide from the capsid of HCV. For the assay 50 µl of sample and controls were incubated at 37 ^{0}C for 90 minutes, with 100 µl of conjugate 1 (biotinylated anti-HCV capsid monoclonal antibody). After a cycle of washing steps, 100 µl of conjugate 2 (peroxidase-labeled antibodies to human immunoglobulin G and streptavidin-peroxidase) was added to each well and incubated for 30 minutes at 37 ^{0}C. After subsequent washing steps, the antigen-antibody

complex was revealed by the addition of substrate. The reaction was stopped after 30 minutes of incubation at room temperature by adding a stop solution. The OD was read at 450/620 nm using the same microplate reader. The cut-off value was determined as the mean of positive control OD divided by 4.

3.2.4 Data analysis and interpretation

Data entry and analyses were performed using GraphPad Prism 7 (GraphPad Software). All samples were tested in duplicate and the test of samples in both assays was performed simultaneously. A sample was considered as positive when the mean difference *(*± standard deviations*)* in the OD value between the sample and the cut-off was greater or equal to zero.

3.3 METHODS (PART II)

3.3.1 Study participants

For the evaluation of an in-house HCV recency assay, the DS/PS specimens were prepared from primary or follow-up samples collected from: (i) the national multicenter long-term observational German HIV-1 Seroconverter (SC) Study at the Robert Koch Institute (Machnowska *et al.*, 2017, Machnowska *et al.*, 2019) (ii) a Commercial HCV seroconversion panel (PHV920(M); SeraCare, MA, USA) (iii) a panel provided by the Institute of Virology, University Duisburg-Essen, Essen, Germany (iv) a panel provided by the Medizinisches Infektiologiezentrum Berlin, Berlin, Germany. Participants of the German HIV-1 Seroconverter (SC) Cohort gave signed informed consent. The study was approved in 2005 and reapproved in 2013 by the Ethics Committee of the Charité, University Medicine Berlin (EA2/105/05). For seroconverters from the SC Cohort and from the panel obtained from the MedizinischesInfektiologiezentrum Berlin, the date of the last negative test result was available and the date of infection was calculated as the arithmetic mean from the last negative and a first positive documented HCV-antibody test result. Only patients with a maximal time interval of 90 days between the last negative and the first positive HCV-antibody test results were included in the study to keep the infection date as precise as possible. Additional information regarding HIV status, sex and age of the HCV seroconversion panels were provided by the diagnostic laboratories at the time of sample collection. HCV genotype was determined as described by (Wang *et al.*, 2019).

3.3.2 Evaluation panel

To evaluate the avidity assay we used three evaluation panels: panel 1 consisted of primary and at least one follow-up samples to analyze the temporal changes of AIs over time in genotype 1 (n=170) and non-genotype 1 infections (n=48; total=218). One aim of this analysis was to find the optimal cut-off value for the avidity assay, i.e. the cut-off value that optimizes the accuracy of the assay. For an AI below that cut-off value, the assay gives a positive result and the infection is classified as recent. Therefore, a sub-panel (1a) of longitudinal DS/PS obtained from individuals with primary and at least two follow-up samples (18 individuals; 2-6 specimens per individual; total=66) was used to estimate the probability that a sample is classified as recent as a function of the known duration of infection by Mixed-effects logistic regression for an optimal AI cut-off. The fixed part of the model was described by (Brookmeyer *et al.*, 2013) and the random effects accounted for multiple samples contributed by the same individual. Since we could only analyze data of 18 patients, we did not include

the cubic terms in the regression model. We calculated the `Mean Duration of Recent Infection´ (MDRI) by numerical integration assuming that this probability is zero for durations longer than 2 years. Another sub-panel (1b) constituting a follow up of acute cases (<26 weeks; `recent infection sample set') was used to calculate the false long-term infection rate (FLTR) (identical to 100 minus sensitivity).

Panel 2 comprising DS/PS samples obtained from 132 individuals with chronic HCV infection who were anti-HCV IgG positive and HCV RNA positive for more than 2 years (>104 weeks; 'long-term infection sample set') was used to calculate the false recent rate (FRR) (identical to 100 minus specificity). Both FLTR and FRR were calculated for all genotypes and separately for genotype 1 and non-genotype 1 infections, for AI cut-off values from 10% to 45% with an increment of 5%.

Panel 3 consisting of 32 samples with a resolved infection and a minimum follow-up of 180 days after clearing the infection (range: 259-2,863 days) was also used to calculate the FRR.

3.3.3 Dried serum/plasma spots

The assay was established and evaluated for the use of DS/PS. For preparation of DS/PS plasma or serum samples (100 µl) were dropped on Whatman 903 filter cards (GE Healthcare Bio-Sciences Corp), air dried overnight at room temperature packaged in a zip-lock bag containing desiccant packets and stored at -20°C immediately for approximately one month before tested. For application, antibodies were eluted from spots in 500 µl elution buffer (500 µl PBS, 0.05% Tween, 3% FCS) resulting in a 1:5 dilution for the avidity assay.

3.3.4 Modified BioRad avidity assay

The HCV immunoglobulin G (IgG) antibody avidity assay was developed by using a Monolisa Anti-HCV PLUS Version 3 ELISA kit (BioRad) and a modified protocol from Patel and coworkers (Patel *et al.*, 2016). Patel and colleagues utilized in their study the Ortho HCV Version 3.0 ELISA (Ortho Clinical Diagnostics) test system with 10 µl of specimen in 200 µl of sample diluent and performed an initial sample incubation of 30 minutes at 4°C. In contrast, in this study DS/PS eluates were applied in duplicate on antigen-coated wells (50 µl of specimen in 100 µl of sample diluent) and incubated overnight at 37°C. We optimized the duration of sample incubation and temperature in a preliminary test to increase the amount of antibodies bound to the plate before treatment (expressed by higher OD values in washing

buffer treated wells, defined to be >1 and <4) in order to optimize the differentiation between recent and long-term infections. In addition, one recent and one long-standing sample with known duration of infection each (≤3 months and ≥2 years of infection, respectively) were used as internal test controls. In agreement with Patel and colleagues, for the dissociation step the first well of each sample was manually treated with 200 µl of 0.025M diethylamine (DEA; diluted with wash buffer) to dissociate antigen antibody complexes and the second well of the same sample was treated with wash buffer as a control. The plate was then incubated for 30 minutes at 37°C, followed by washing with wash buffer (300 µl) 5 times. The remainder of the test (beginning with the addition of conjugate) was performed according to the manufacturer's instructions. Samples treated with wash buffer with an OD ≤1.0 were excluded from the study. We did not find samples with OD ≥4.0 (limit of detection on the plate reader).

3.4 METHODS (PART III)

3.4.1 Clinical samples

According to the "Protection against Infection Act" (IfSG; §7) of 2001, newly diagnosed HIV infections are anonymously reported to the German public health institute (Robert Koch Institute, RKI). For surveillance programs a network of approximately eighty diagnostic laboratories was established that send along with the report form, residual serum from newly diagnosed HIV cases spotted onto a filter card (Whatman 903 filter cards, GE Healthcare Bio-Sciences Corp) as DSS. According to §13 of IfSG, the RKI is authorized to receive blood residuals from diagnostics for surveillance purposes. By this sampling strategy, specimens from approximately 60% of all reported newly diagnosed HIV infections are send to the RKI laboratory. HIV notifications and DSS samples were linked by using the number on the HIV notification form. Data on age, gender, mode of transmission, region of origin and assumed region of infection were used from the associated HIV notification form. Exclusion criteria were unknown information on gender or age, age below18 years, or DS/DP samples from double-notified HIV cases. Ethical approval for the nationwide cross-sectional study was given by the ethics board at the Charité, University Medicine, Berlin, as well as approval from the data protection office of Germany according to the Federal Data Protection Act. The DSS samples used were residuals from routine HIV diagnostic processing; therefore, no patient informed consent was given (Hauser *et al.*, 2018).

3.4.2 Workflow of the project

DSS from newly reported HIV infections with a date of diagnosis between the diagnoses years 2015 and 2017 were analyzed for the presence of anti-HCV antibodies and/or antigen by means of ELISA (Monolisa HCV Ag-Ab ULTRA V2, Bio-Rad). ELISA-reactive (ELISA-positive) samples were further characterized into active infections (HCV seropositive + HCV RNA-positive) or resolved infections (HCV seropositive + HCV RNA-negative) by an in house real-time RT-PCR (qPCR) targeting the 5'UTR of the HCV genome. Furthermore, qPCR positive samples were classified into recently acquired and long-term HIV-infections using the already established an in-house recency assay. On the other hand, qPCR-negative samples were further analyzed by an Immunoassay (MIKROGEN, 2019) to identify false positive samples in the initial screening ELISA and to define the true proportion of HCV positive samples (HCV seropositive and/or HCV-RNA positive). Genotyping (based on NS5B region) and resistance associated substitutions (in the NS3, NS5A, and NS5B regions)

were further carried out by Nested-PCR followed by Sanger/Next Generation sequencing (Fig. 7).

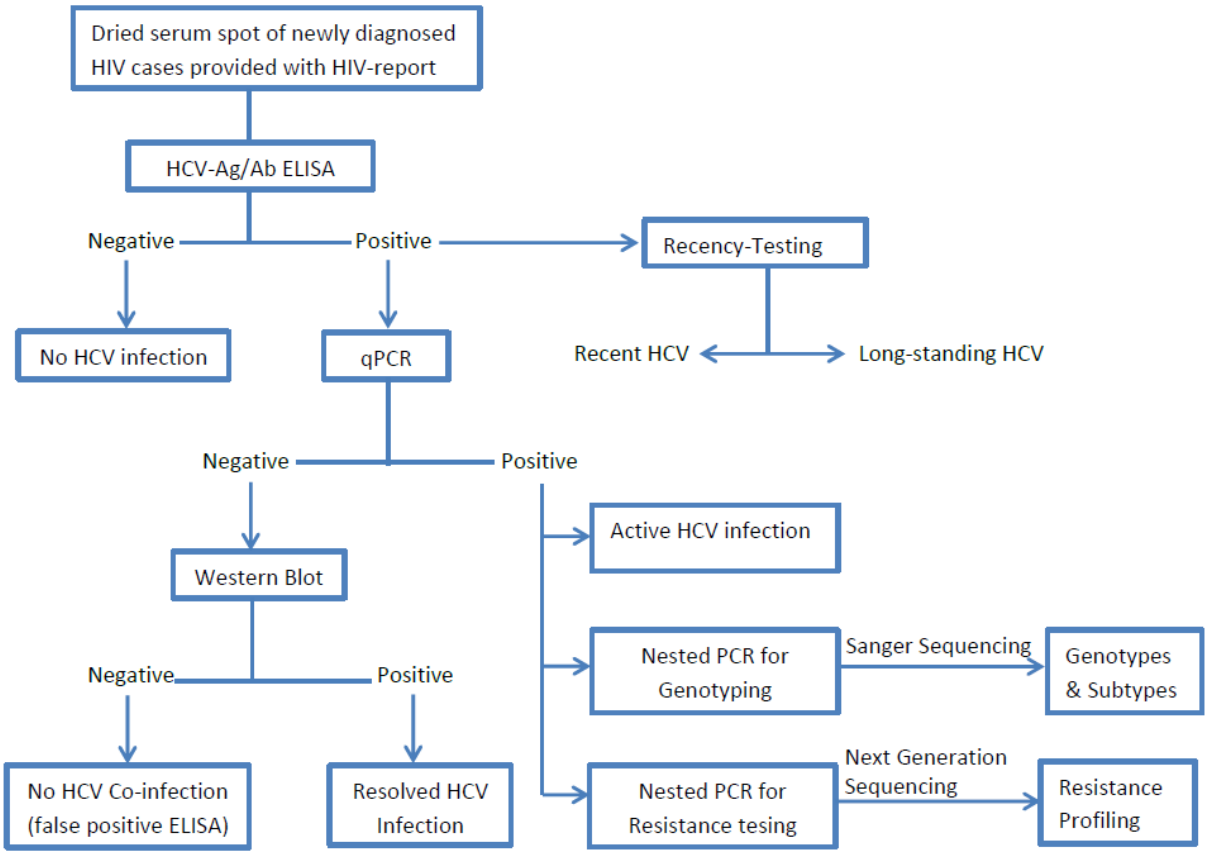


Fig. 7: Workflow of the project.

3.4.3 The Pre-DAA study

Analysis of HCV coinfection among newly diagnosed HIV cases in prior to the introduction of HCV-specific antiviral drugs (pre-DAA) in Germany was performed by Johanna Riege (Bachelor thesis) (Riege *et al.*, 2019). The aim of this bachelor thesis was to gain an overview of HCV coinfections in HIV new diagnosis before the introduction of DAA therapy in the diagnoses years 2009-2011. The data from the pre-DAA study was taken for comparison with the current study (2015-2017) to assess the potential impact of DAA therapy in terms of disease outcomes and HCV subtype distribution in Germany.

3.4.4 Laboratory testing

3.4.4.1 ELISA

DSS were screened using a commercially available antibody/antigen ELISA (Monolisa Ag-Ab Ultra V2, BioRad) according to the manufacturer's instructions (see section 3.2.3.2).

3.4.4.2 Nucleic acid (RNA) extraction

RNA was semi-automatically extracted using the NucliSENS® easyMAG platform (BioMérieux) according to the manufacturer`s instructions. Accordingly, 2 DSS (each with 100 µl serum) were placed in a 2 ml Eppendorf tubes and mixed with 1.8 ml Lysis buffer. The samples were then shaken for one hour at about 300 rpm and room temperature. Thereafter, 1.5 ml of the lysed eluate was withdrawn and transferred to a disposable reaction vessel. After adding 50 µl of magnetic silica solution, the samples were mixed with an automatic pipette and positioned in the extractor. The device was then set and started as described in the instruction (protocol: Generic 2.0.1, quantity: 0.4ml, eluate: 60µl, sample type: serum). RNA was extracted by automated magnetic separation and finally eluted in 60 µl of elution buffer (Fig. 8). The Extracted RNA was aliquoted in portions of 10 µl and immediately stored at -80°C until further use.

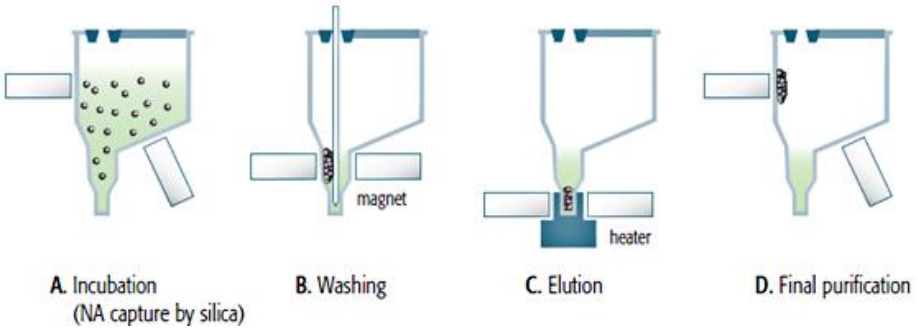


Fig. 8: Principle of nucleic acid extraction with the Biomerieux NucliSense® easyMAG® (BioMérieux, 2019).

A) During incubation of the lysed samples, all the target nucleic acid is captured by magnetic silica particles. B) The NucliSens® easyMAG® magnetic device attracts all the magnetic silica, enabling the system to purify the nucleic acids through several washing steps. C) The heating step releases the nucleic acids from the silica. D) At the final step, the magnetic silica particles are separated from the eluate by the magnetic device.

3.4.4.3 RT-qPCR

The 5'UTR is a highly conserved region of the HCV genome and is targeted by most diagnostic assays for HCV RNA. Based on the consensus sequence of this region, an in-house quantitative real-time PCR (qPCR) was used for viral quantification and for distinguishing active and resolved HCV infections. To prepare the Master Mix, the PCR kit SuperScript® III Platinum® One-Step qRT-PCR Kit (ThermoFisher GmbH) was used. As described in table 2, 4µl from each of the Master Mix and 8µl of the extracted RNA were distributed into a 96 well

plate. Standards (plasma samples of HCV genotype 1a in concentration 10E+01-10E+06 international units (IU)/ml) and negative controls (HCV-negative plasma) were also analyzed in each run.

Table 2: Master Mix composition for the RT-qPCR.

RT-qPCR			**Master Mix**
Reagents	Stock concentration	Final concentration	µl/sample
Water			0.6
2x SSIII/PlatTaq RT-PCR MM			6
Primer HCV-255	10µM	416nM	0.5
Primer HCV-256	10µM	416nM	0.5
ProbeHCV-TM5	10µM	167nM	0.2
Enzyme-Mix			0.2
∑			8
+Template (RNA)			4

After a short centrifugation, the plate was placed in the Lightcycler 480 II (Roche) and the samples were amplified according to the program in table 3. Each sample was analyzed in duplicate and the mean concentration per samples was extrapolated using the standard curve.

Table 3: Cycler program for the RT-qPCR.

Steps	**Temperature**	**Time**	**Cycle**
cDNA Synthesis	50°C	15 min	1
Denaturation	95°C	2 min	1
Denaturation	95°C (ΔR 3°C/s)	15 sec	45
Annealing and Elongation	58°C (ΔR 1°C/s)	45 sec	45

3.4.4.4 cDNA synthesis

In order to further characterize the viral subtypes and drug resistance profiling of the samples identified as active infection (RNA positive) by the qPCR, the extracted RNA was transcribed into cDNA and subsequently amplified in a nested PCR for further use in a genotyping and resistance testing PCRs. First, the RNA was denatured for 4 minutes at 65°C. Meanwhile, the

Master Mix was prepared as described in table 4. Then 10µl of the sample and 10µl of the mix were pipetted together and mixed by up and down pipetting.

Table 4: Master Mix composition for cDNA synthesis.

cDNA			**Master Mix**
Reagents	Stock concentration	Concentration final	µl/sample
RT-Puffer	5x	1x	4
dNTP	2.5mM	0.5mM	4
AS Primer R6	25µM	0.5µM	0.4
DTT	100mM	5mM	1
RNasin (Promega)	40U/µl	20U	0.5
SII (Invitrogen)	200U/µl	100U	0.4
∑			10.4

The cDNA synthesis was then carried out according to the following protocol in table 5. After synthesis, the cDNA was stored at -25° C until use.

Table 5: Cycler program for cDNA synthesis.

Steps	**Temperature**	**Time (min)**
Synthesis	42°C	60
Heat inactivation	93°C	7
	4°C	Pause

3.4.4.5 Western blot

The recomLine HCV IgG is a line immunoassay (Western blot) that serves to detect antibodies present in a sample for qualitative detection of antibodies against individual antigens of HCV and is used as confirmatory test if a previous antibody screening test (Monolisa Ag-Ab Ultra V2, BioRad) was reactive. The test principle allows for separate lining up of the individual antigens and thus, unlike ELISA, for the identification of specific antibodies against the individual HCV antigens (Core 1, Core 2, Helicase, NS3, NS4, and NS5). In the first step, the test strip was incubated with DSS eluate (20µl sample at 2ml wash buffer per strip) in a dish for 3 hours with gentle shaking. In this case, the existing HCV antibodies from the samples bind to the antigens fixed on the test strips. The unbound antibodies were then removed by washing 3 times with a wash buffer. In the second step, 2ml

of peroxidase conjugated anti-human antibodies (IgG specific) were added and incubated for 45 minutes while shaking gently. The unbound antibodies were removed by washing 3 times with washing buffer. Finally, 1.5ml of a ready-to-use substrate solution was added and incubated for 8 minutes with gentle shaking. The reaction was then stopped by washing the strips at least three times with deionisied water. If an antigen-antibody reaction has taken place, a dark band appears on the strip at the corresponding point. Analysis of the bands on the test strips was done on the following day by using the instructions found in the kit insert (MIKROGEN, 2019).

3.4.4.6 Nested PCR for genotyping and subtyping

For the genotyping and subtyping of HCV, both sequences from the 5'UTR region and the NS5B region of the HCV genome can be used. However, since the 5'UTR range is highly conserved, it is difficult to distinguish between individual subtypes. In particular, subtypes 1a and 1b are difficult to differentiate because they differ only in a single base at position -99 in the 5'UTR (Chen and Weck, 2002). Due to its variable nucleotide sequence and based on the consensus criteria used by Smith *et al* (Smith *et al.*, 2014), the NS5B region has become particularly well suited region for the identification of both HCV subtypes and DAA mutations.

However, due to the use of DSS, RNA degradation needs to be considered for the choice of an appropriate PCR-assay, especially the size of the amplicon should not exceed 900bp (experience from our laboratory). For this region, the resistance associated positions are spread over a length of 1200 bp and; therefore, the use of 2 primers for amplifying regions of DAA resistance is a problem. Hence, two sets of primers ("long" genotyping part I and NS5B part II) were designed and established (Fig. 9). The "short" genotyping is used as a backup PCR for amplifying samples which were negative in the "long" genotyping PCR.

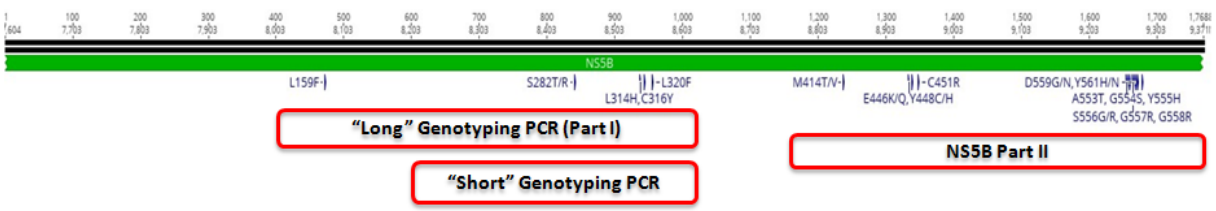


Fig. 9: Regions of the NS5B used for identification of HCV genotypes/subtypes and DAA associated mutations.

In case that both PCR-assays were negative a two-step nested-RT-PCR with primers lying in the 5'UTR was used, in order to obtain a subtype for as many samples as possible.

In the case of long nested PCR, the external primers 271 (sense), 275 (antisense) and 305 (sense) were used to amplify a 685bp PCR product (Table 18). For this, 11.5 µl of the prepared Master Mix (Table 6) was mixed with 1 µl of the cDNA template and amplified according to the program below (Table 7).

Table 6: Master Mix composition for the first round NS5B "long" nested PCR.

1.PCR			**Master Mix**
Reagents	Stock concentration	Concentration final	µl/sample
Water			0.875
Q-Solution	5x	1x	2.5
Primer HCV-271	10µM	400nM	0.5
Primer HCV-305	10µM	400nM	0.5
Primer HCV-275	10µM	400nM	0.5
Bovine Serum Albumin (BSA)	20µg/µl	0,6µg/µl	0.375
HotStart Master Mix	2x	1x	6.25
∑			11.5
+Template (cDNA)			1

Table 7: PCR cycler program for the first round NS5B "long" nested PCR.

Steps	**Temperature**	**Time**	**Cycle/s**
First Denaturation	95°C	15min	1
Denaturation	94°C	10sec	10
Primer Annealing	60°C (ΔT -1°C, ΔR 1°C/s)	30sec	10
Elongation	72°C	50sec	10
Denaturation	94°C	10sec	30
Primer Annealing	52°C (ΔR 1°C/s)	30sec	30
Elongation	72°C	50sec	30
Elongation	72°C	2min	1
	16°C	Pause	

The second round long-nested PCR was carried out with primers 272 (sense) and 275 (antisense) resulting a final PCR-product of 674bp (Table 18). For this, 0.75µl was taken from the final PCR product and mixed with a second Master Mix (Table 8) and re-amplified as per the program in table 9.

Table 8: Master Mix composition for the second round NS5B "long" nested PCR.

2.PCR			**Master Mix**
Reagents	Stock concentration	Concentration final	µl/ sample
Water			5
Primer HCV-272	10µM	200nM	0.25
Primer HCV-275	10µM	200nM	0.25
HotStart Master Mix	2x	1x	6.25
∑			11.75
+Template (1.PCR)			0.75

Table 9: PCR cycler program for the second round NS5B "long" nested PCR.

Steps	**Temperature**	**Time**	**Cycle/s**
First Denaturation	95°C	15min	1
Denaturation	94°C	10sec	30
Primer Annealing	52°C (ΔR 1°C/s)	30sec	30
Elongation	72°C	50sec	30
Elongation	72°C	2min	1
	16°C	Pause	

For the first round short-nested PCR, the primers 257 (sense) and 270 (antisense) were used to produce a PCR product of 392bp (Table 18).The primer pairs were mixed with 11.5µl of the prepared Master Mix (Table 10) and mixed with 1µl of the cDNA template. The amplification was carried outaccording to the program below (Table 11).

Table 10: Master Mix composition for the first round NS5B "short" nested PCR.

1.PCR			**Master Mix**
Reagents	Stock concentration	Concentration final	µl/ sample
Water			1
Q-Solution	5x	1x	2.5
Primer HCV-257	10µM	400nM	0.5
Primer HCV-270	10µM	400nM	0.5
BSA	20µg/µl	0.6µg/µl	0.75
HotStart Master Mix	2	1x	6.25
∑			11.5
+Template (cDNA)			1

Table 11: PCR cycler program for the first round NS5B "short" nested PCR.

Steps	**Temperature**	**Time**	**Cycle/s**
First Denaturation	95°C	15min	1
Denaturation	94°C	10sec	10
Primer Annealing	60°C (ΔT -1°C, ΔR 1°C/s)	30sec	10
Elongation	72°C	40sec	10
Denaturation	94°C	10sec	30
Primer Annealing	52°C (ΔR 1°C/s)	30sec	30
Elongation	72°C	40sec	30
Elongation	72°C	2min	1
	16°C	Pause	

After the first round short nested PCR, 0.75 µl of the PCR product was transferred to 11.75 µl of a new Master Mix (Table 12) which contains primers 257 (sense), 259 (sense) and 270 (antisense) (Table 18). The final mix was re-amplified as the program in table 13.

Table 12: Master Mix composition for the second round NS5B "short" nested PCR.

1.PCR			**Master Mix**
Reagents	Stock concentration	Concentration final	µl/ sample
Water			5
Primer HCV-259	10µM	200nM	0.25
Primer HCV-270	10µM	200nM	0.25
HotStart Master Mix	2	1x	6.25
∑			11.75
+Template (cDNA)			0.75

Table 13: PCR cycler program for the second round NS5B "short" nested PCR.

Steps	**Temperature**	**Time**	**Cycle/s**
First Denaturation	95°C	15min	1
Denaturation	94°C	10sec	30
Primer Annealing	52°C (ΔR 1°C/s)	30sec	30
Elongation	72°C	30sec	30
Elongation	72°C	2min	1
	16°C	Pause	

For the first round 5'UTR nested PCR, primers 238 (sense) and 239 (antisense) were used to produce a product of 262bp (Table 18). The primer pairs were mixed with 11.5µl of the prepared Master Mix (Table 14) which was then mixed with 1µl of the cDNA template. The final mix was amplified according to the program below (Table 15).

Table 14: Master Mix composition for the first round 5'UTR nested PCR.

1.PCR			**Master Mix**
Reagents	Stock concentration	Concentration final	µl/ sample
Water			3.5
Primer HCV-238	10µM	400nM	0.5
Primer HCV-239	10µM	400nM	0.5
BSA	20µl	0,6µg/µl	0.75
HotStart Master Mix	2x	1x	2x
∑			11.5
+Template (cDNA)			1

Table 15: PCR cycler program for the first round 5'UTR nested PCR.

Steps	Temperature	Time	Cycle/s
First Denaturation	95°C	15min	1
Denaturation	94°C	10sec	40
Primer Annealing	56°C (ΔR 1°C/s)	30sec	40
Elongation	72°C	30sec	40
Elongation	72°C	2min	1
	16°C	Pause	

For the second round 5'UTR nested PCR, primers 235 (antisense) and 240 (sense) were used resulting in a product length of 215bp (Table 18). For this, 0.75µL of the first PCR product was mixed with 11.75 µl of the prepared Master Mix (Table 16) and re-amplified according to the program in table 17.

Table 16: Master Mix composition for the second round 5'UTR nested PCR.

2.PCR			Master Mix
Reagents	Stock concentration	Concentration final	µl/sample
Water			5
Primer HCV-235	10µM	200nM	0.25
Primer HCV-240	10µM	200nM	0.25
HotStart Master Mix	2x	1x	6.25
∑			11.75
+Template (1.PCR)			0.75

Table 17: PCR cycler program for the second round 5'UTR nested PCR.

Steps	Temperature	Time	Cycle/s
First Denaturation	95°C	15min	1
Denaturation	94°C	10sec	35
Primer Annealing	58°C (ΔR 1°C/s)	30sec	35
Elongation	72°C	30sec	35
Elongation	72°C	2min	1
	16°C	Pause	

Table 18: Details of the primers used for qPCR, NS5B ("long"), NS5B ("short"), and 5′UTR PCRs.

Primer	Sequence 5′-3′	PCR-product (bp)	Position*
qPCR-5'UTR			
HCV-255-F	AGY GTT GGG TYG CGA AAG	54	260-278
HCV-256-R	CAC TCG CAA GCR CCC T		298-314
HCV_TM-5	FAM 5′CCT TGT GGT ACT GCC TGA 3′MGB		279-297
Nested-PCR-NS5B ("long")			
HCV-271-F	ACC ACA TCM RST CCG TGT GG	Outer PCR:	7951-7971
HCV-272-F	TCC GTG TGG RAR GAC YTS CTR GA	685	7962-1985
HCV-275-R	CTS GTC ATA GCY TCC GTG AA	Inner PCR:	8616-8636
HCV-305-F	CTC CGT MTG GGA GGA CTT GC	674	7691-7971
Nested-PCR-NS5B ("short")			
HCV-257-F	GGS TTY TCN TAT GAY ACC MGV TGY TTT GA	Outer PCR: 392	8247-8276 8266-8288
HCV-259-F	GCT GYT TTG AYT CAA CNG TCA C	Inner PCR:	8614-8639
HCV-270-R	TAY CTG GTC ATA GCY TCC GTG AAR G	373	
Nested-PCR-5'UTR			
HCV-235-R	AGT ACC ACA AGG CCT TTC G	Outer PCR:	49-69
HCV-238-F	GAG GAA CTA CTG TCT TCA CG	262	272-291
HCV-239-R	TCG CAA GCA CCC TAT CAG	Inner PCR:	293-311
HCV-240-F	CGT CTA GCC ATG GCG TTA G	215	76-95

F=forward; R=reverse; *Position according to HCV reference genome HCV-1a (AF009606).

3.4.4.7 Sanger-sequencing and evaluation

Samples which were positive in one of the three (NS5B-"long", NS5B-"short" or 5'UTR) nested-RT-PCR assays were considered for genotyping/subtyping and were further sequenced using the BigDye Terminator v3.1 cycle Sequencing Kit. First, 5µl of PCR-product were first purified using 2µl of ExoSAP-IT (cycle program: 15 mins at 37°C., then 15 mins at 80°C). Then, 9µL of the prepared Master Mix was mixed with 1µL of the purified PCR products (Table 19) and amplified according to the cycler program shown in table 20. As for sequencing primers, the respective inner primers of the nested PCR were used. Hence, for PCR-products positive in the NS5B-"long" nested-PCR, primers HCV-259-F, HCV-272-F and HCV-275-R were used. For PCR-product generated by NS5B-"short" nested-PCR, primers HCV-257-F, HCV-259-F and HCV-270-R were used. For sequencing of PCR-

product obtained by 5'UTR-nested-PCR, primers HCV-235-R and HCV-240-F were used (Table 18). The sequencing took place in the in-house sequencing laboratory.

Table 19: Master Mix composition for Sanger sequencing.

Reagents	µl/sample
Water	6
Primer (10µM)	0.5
5x Buffer	2
BigDye 3.1	0.5
∑	9
+Template	1

Table 20: PCR cycler program for Sanger sequencing.

Steps	Temperature	Time	Cycle/s
First Denaturation	96°C	1min	1
Denaturation	96°C	10sec	25
Primer Annealing	52°C (ΔR 1°C/s)	30sec	25
Elongation	60°C	4min	25
	16°C	Pause	

Due to the fact that HCV is present as so-called quasispecies, there is a population of closely related but different viruses. Thus, different bases can occur at one and the same position in the genome (ambiguities). Therefore, manual correction of the raw sequence data was done by using Geneious® 11.1.5 (Biomatters), aligned to a reference sequence (HCV-1a_AF009606), and trimmed by their quality. The sequences were then checked for the presence of ambiguous positions using the IUPAC-IUB codes and the consensus-sequences were saved as fasta-file. The subtypes were determined using geno2pheno, a web-tool offering detailed sub-genotyping of HCV as well as analysis of DAA-susceptibility for each drug target (Kalaghatgi *et al.*, 2016). For samples sequenced by the 5'-UTR nested PCR, the HCV phylogenetic typing tool 2.4 (GenomeDetective) and the NCBI genotyping tool (Rozanov *et al.*, 2004), both available as a web tool, were used to determine the subtype. If different subtypes were identified for a sequence in the two web tools, a clear classification of the subtype was not possible and the genotype was only used for data analysis. Due to a lack of sequence variability, subtyping in the 5'UTR is not always possible (Chen and Weck, 2002).

3.4.4.8 Nested PCR for drug resistance mutations

Due to the degradation of RNA on DSS and the high divergence of HCV, the design of genotype-and even subtype specific primers was necessary to cover resistance associated positions in the NS5B, NS3, and NS5A regions of the genome. For this reason, an alignment with 204 sequences of all HCV subtypes (available from the Los Alamos HCV database) was generated. The drug resistance mutations were identified based on Pawlotsky *et al* (Pawlotsky, 2016) with a list of amino acid substitutions reported to reduce susceptibility of different HCV genotypes or subtypes to DAAs. According to this, primers were designed for NS3, NS5A, and NS5B regions of genotype 1 (subtypes 1a and 1b) and NS5A region of genotype 2 (all subtypes), genotype 3 (subtype 3a), and genotype 4 (all subtypes) (Fig. 10). However, since genotype 5, 6 and 7 are mostly prevalent in Africa and Asia; primers were not designed for these genotypes.

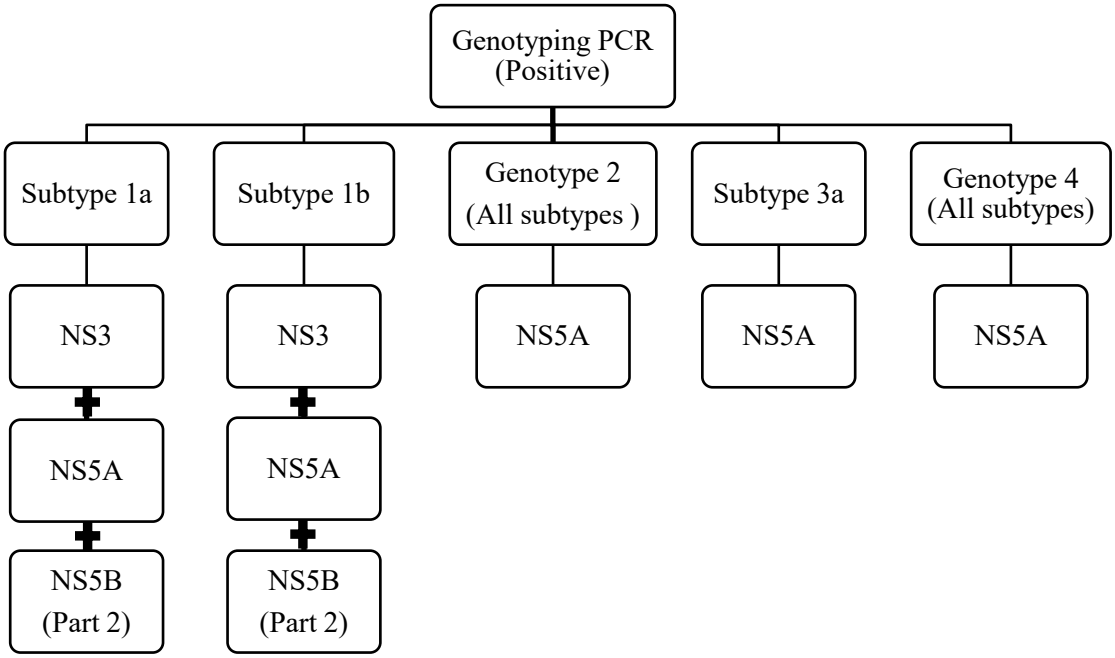


Fig. 10: Workflow for detection of resistance mutations against DAAs in different HCV subtypes.

Tables 21 and 22 show the different primer combinations used to cover resistance associated mutations in the NS3, NS5A and NS5B regions of different subtypes. The alternative PCRs were used as a backup PCR for amplifying samples which were negative in the standard PCRs. For all HCV subtypes of the first round nested PCR, 0.5µl of the primer pairs (400nM) were mixed with 3.9µl of water, 0.375µl of BSA (0.6µg/µl) and 6.25µl of Hot Start Master

Mix to prepare a Master Mix volume of 11.5µl, which was finally mixed 1µl of the cDNA template. For the second round nested PCR, 0.25µl of the primer pairs (200nM) were mixed with 5µl of water and 6.25µl of Hot Start Master Mix with a final Master Mix volume of 11.75µl. The Master Mix was then mixed with 1µl of the product from the first round PCR.

For the first round standard nested PCR, an initial denaturation step of 15min at 95ºC was used in all programs, followed by a second denaturation of 10sec at 94ºC repeated for 10 cycles. The annealing step was performed for 45sec for NS3 region of subtype 1a at 60°C (ΔT -1°C, ΔR 1°C/s)], subtype 1a (alternative) and subtype 1b at 50°C (ΔT +0.4°C, ΔR 1°C/s), NS5A region of subtypes 1a, 1b, and 3a and for NS5B region of subtypes 1a and 1b at 50°C (ΔT +0.5°C, ΔR 1°C/s) and then an elongation step of 15sec at 72°C. Both steps were repeated for 10 cycle and the subsequent 30 cycles were performed for 10sec at 94°C, 45sec at 55°C and 45sec at 72°C. Finally, an elongation period of 2min at 72°C was performed in Biometra Trio thermocycler (Biometra GmbH).

The second round nested PCR was performed for all programs using an initial denaturation step of 15 min at 95°C and a second denaturation of 15 sec at 94°C. The annealing step was performed for 45 sec for NS3 region of subtype 1a at 54°C (ΔR 1°C/s), subtype 1a (alternative) at 52°C (ΔR 1°C/s) and subtype 1b at 50°C (ΔT +0.5°C, ΔR 1°C/s), NS5A region of subtype 1a at 54°C (ΔT +0.5°C, ΔR 1°C/s), subtypes 1a, 1b and 3a at 50°C (ΔT +0.5°C, ΔR 1°C/s) and for NS5B region of subtypes 1a and 1b at 50°C (ΔT +0.5°C, ΔR 1°C/s) and then an elongation step of 45 sec at 72ºC. After repeating both steps for 10 cycles, a second stage of 25 cycles was followed for 10sec at 94°C, 45sec at 55°C (ΔR 1°C/s) and 45sec at 72°C (ΔR 1°C/s). An elongation step of 2 min at 72ºC was performed in all programs after stage 2.

Table 21: Primer combinations used for PCRs in the NS3, NS5A and NS5B regions of different HCV subtypes.

Genome Region	HCV Subtype	1st PCR Primers (standard)	1st PCR Primers (Alternative)	2nd PCR Primers (Standard)	2nd PCR Primers (Alternative)
NS3	1a	261 + 262	263 + 281	263 + 264	280 + 281
	1b	265 + 266	-	265 + 283	267 + 268
NS5A	1a	284 + 286	-	285 + 286	-
	1b	308 + 309	-	289 + 291	-
	3a	292 + 294	292 + 293 + 294	293 + 295	292 + 293 + 295
	2a/b	335 + 336	-	335 + 337	-
	4a/d	362 + 364	-	363 + 364	-
NS5B	1a	297 + 311+ 298	-	296 + 299	-
	1b	301 + 303	-	301 + 302	-

Table 22: Details of the primers used for PCRs in the NS3, NS5A and NS5B regions of different HCV subtypes.

Genome region/Subtype	Sequence 5'-3	Lengthouter/inner (bp)	Position
NS3-1a	261FGAT CTG GCC GTG GCT GTA GAG 262R AGC ACC TTG TAG CCC TGA GC 263F GCG GTG ACA TCA TCA ACG GCT 264RGGG ACC TCA TGG TTG TCT CTA GG **Alternative PCR** 263F GCG GTG ACA TCA TCA ACG GCT 280F GGA GGT TGC TGG CGC CCA T 281R GCC GGG ACC TTG GTG CTC TT	860/645 748/658	*[1] 3237-4097 *[1] 3319-4066
NS3-1b	265FCTT GCG GTG GCA GTT GAG C 266RCCG TGG TGA TGG TCC TTA CCC 265FCTT GCG GTG GCA GTT GAG C 283R GCG TGT AGA TGG GCC ACT TG **Alternative PCR** 265FCTT GCG GTG GCA GTT GAG C 266RCCG TGG TGA TGG TCC TTA CCC 267F GCT GCA TCA TCA CCA GCC TCA C 268R GGC ACC TTA GTG CTC TTG CC	964/824 964/601	*[2]3228-4192 *[2]3228-4191
NS5A-1a	284FCAT ACT CAG CAG CCT CAC TGT 286R CCA TAT AAC AGC AGA GGC GGC 285F ACC AGT GGA TAA GCT CGG AGT G 286RCCA TAT AAC AGC AGA GGC GGC	720/676	*[1] 6179-6899
NS5A-1b	308F ATG AAC CGG CTG ATA GCG TTC 309R GCTCACTTCCATGCTYACCGA 289FGCA CTA TGT GCC TGA GAG CG 291R CAG TGC TCA CTT CCA TGC TCA	785/731	*[2] 6075-6859
NS5A-3a	292FGCT GAG TTC TCT AAC TGT CAC AAG 294R TCC ATC AGA GGC AAG CTC ATC 293FACT GTC ACA AGT CTG CTC CGG 295RGCT GAC CTC GAT GTT GAG AGA CC **Alternative PCR** 292FGCT GAG TTC TCT AAC TGT CAC AAG 293FACT GTC ACA AGT CTG CTC CGG 294R TCC ATC AGA GGC AAG CTC ATC 295RGCT GAC CTC GAT GTT GAG AGA CC	765/679	*[3] 6198- 6962
NS5A-2a/b	335FAAA CCA TGT TGC CCC TAC CC 336RGTC TGT CAA CAT GGA GGC CA 335F AAA CCA TGT TGC CCC TAC CC 337RGAG AGC CGA CCA CAA AGG AA	754/698	*[4] 6131-6885
NS5A-4a/d	362FRCY ATY GCY TCC CTC ATG 364RGRC AGG GRC ACT TGA BRT T 363FAAG ATC ATG AGY GGY GAG A 364RGRC AGG GRC ACT TGA BRT T	988/745	*[5] 5635-6622
NS5B-1a	297FGCG AGC CTG AGA GCC TTC AC 311F GCGWGCCTGAGAGCCTTCAC 298R CTA CTC CTG CTT GCT GCA GGG 296F AGG CTA TGA CCA GGT ACT CCG 299R TTT GCC TAC TCC TGC TTG CTG	747/720	*[1] 8601- 9348
NS5B-1b	301FCGG AGG CTA TGA CTA GGT ACT C 303RTCA TCG GTT GGG GAG CAG GTA 301FCGG AGG CTA TGA CTA GGT ACT C 302RGGA GCA GGT AGA TGC CTA CC	758/747	*[2]8605-9362

* (Acc.No. AF009606); *[2] (Acc.No. D90208); *[3] (Acc.No.D28917); *[4] (Acc.No.D10988); *[5] (Acc.No.Y11604).

3.4.4.9 Agarose gel electrophoresis

The success of all PCRs was checked by applying the PCR products on an agarose gel. This has served as an initial assessment of whether the PCR results and a further sequencing of the samples would be possible. A DNA size marker (100bp DNA Ladder (LS100); Invitrogen) was used to determine the size of the PCR product and thus the result of the PCR can be evaluated as positive or negative by a visible band at the correct level. To prepare the gel, 1.5% agarose powder (Carl Roth) was dissolved in 1X Tris-Acetate EDTA (TAE) buffer (Thermo Scientific). Then, the solution was heated in a microwave oven for 2–3 minutes, mixed with 1µl/10ml GelRed (Biotium) and poured into a gel tray with the well comb in place. After allowing it to cool at a room temperature for 10-15 mins, the gel was then put into an electrophoresis chamber and filled with 1xTAE buffer until the gel is covered. Then, 2.5µl of each PCR product and the DNA marker (100bp; Invitrogen.) were mixed with 6X DNA loading dye (bromophenol blue; NEB) and loaded into each well. The gel electrophoresis was performed for 50 minutes at 100 volts/cm. Visualization of the DNA bands was done by the BioDocAnalyze (Biometra GmbH).

3.4.4.10 Purification of PCR products

For subsequent sequencing, it is necessary to free PCR products from nucleotides, salts, and enzymes. For this purpose, the PCR products were purified using the protocol of the Agencourt® AMPure XP Kit (Beckman Coulter). The technology is based on solid-phase paramagnetic beads for high-throughput purification of PCR amplicons. First, the paramagnetic beads are added to the PCR products (ratio 1: 1.8); these then bind the DNA fragments. With the aid of a magnet, the magnetic particles can be separated with the DNA from the remaining components of the preceding PCR. In the two purification steps, the particles are also separated from the ethanol by means of magnets. After elution of the purified PCR products, they can be transferred to a new plate. The described steps of this purification are again shown as an overview in Fig. 11. For the purification, in each case, 20µl of PCR product and 36µl of the magnetic particle reagent were used. The washing steps were carried out in accordance with the Agencourt protocol with 80% ethanol instead of 70%, as a better yield had been shown in a series of previous tests in the working group. Finally, the purified DNA is taken up in 30 µl elution buffer for quantification.

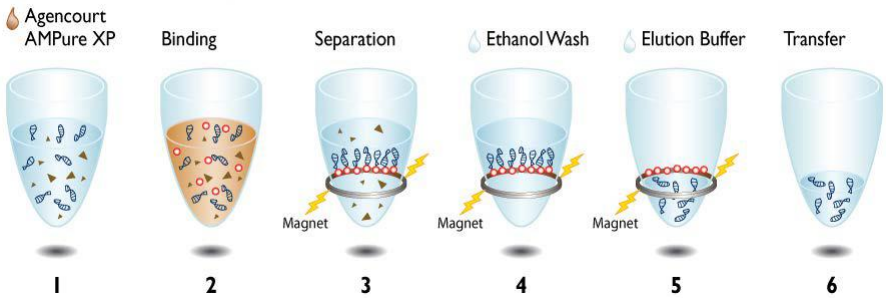


Fig. 11: Scheme of purification of PCR products using Agencourt® AMPure XP (BeckmanCoulter, 2019).

3.4.4.11 Quantification of the purified PCR products

The purified PCR products were quantified photometrically according to the protocol of the Quant-iT™ PicoGreen® kit (Thermoscientific, 2019). First, 100 µl of sample were diluted with Tris-EDTA (TE) buffer (Promega) in a ratio of 1:50 in a black 96-well microtiter plate, with a UV-impermeable film bottom. In addition, a standard series was included in each measurement, which was diluted from a lambda DNA standard (100µg/ml). For quantification, the fluorescent dye PicoGreen was used. This first had to be diluted 1:200 with TE buffer. Subsequently, the samples as well as the standard series and the blank were mixed with 100µl PicoGreen. The fluorescence intensity was measured at 520 nm with a reference wavelength of 480 nm on the Tecan InfiniteF200 reader (Tecan).

3.4.4.12 Next generation sequencing (NGS)

For the sequencing of regions associated with resistance associated mutations (NS3, NS5A, and NS5B), the Next Generation Sequencing method was performed on the Illumina MiSeq® (Illumina, 2019) in accordance with the instructions of the manufacturer. As described in Machnowska *et al* (Machnowska *et al.*, 2017), the NGS data were processed through an in-house bioinformatics pipeline making use of the tools Trimmomatic (version 0.36), FLASH (version 1.2.11), and BWA (version 0.7.15), all wrapped in a Python script. After adapter-clipping, trimming and merging, several iterations of mapping against a personalized constructed reference sequence (which is based on genotypes 1, 2, 3 and 4) were performed. Potential insertions and deletions were considered during the mapping cycles. The final output was subject to further Python scripts, which enabled codon-based variant detection and generation of a consensus sequence containing ambiguities according to an adjustable threshold. For a direct comparison with Sanger sequencing, an NGS limit of 20% was used. Samples with successfully generated sequences were included in the final drug resistance

mutation analysis. After a manual correction of the raw sequence data on Geneious® 11.1.5 (Biomatters), the drug resistance mutations were then determined using the geno2pheno software (Kalaghatgi *et al.*, 2016).

3.4.4.13 Statistical analysis

Descriptive statistical analysis was performed with calculation of means and standard deviation (SD), frequencies and confidence intervals (CI) of 95%. Categorical variables were compared using the Chi-squared test, fisher's exact test and odds ratio when was appropriate. *P*-values were 2-tailed and statistical significance was defined as $p<0.05$. Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA) and MedCalc (version 9.2.1.0, MedCalc Soft-ware, Belgium) software.

4 RESULTS

4.1 RESULTS (PART I)

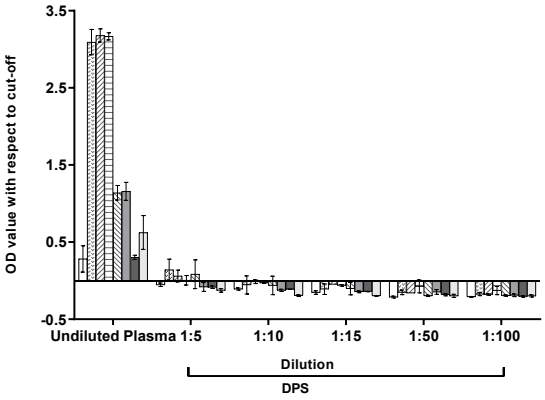
HCV Ag/Ab assays offer the benefit of reducing the window period compared to assays that detect only HCV-Ab. In this study the performance of the Murex Ag/Ab (Murex, Abbott) and Monolisa Ag/Ab Ultra (Monolisa, Bio-Rad) ELISAs was compared for the use of filter dried serum/plasma spots (DS/PS) with a focus on the sensitivity and the percentage of correct positive test results.

The sensitivity of the two assays was first analyzed using the commercially available seroconverter panels. As depicted in Fig. 12A, for HCV subtype 1a plasma reference samples, the Murex became reactive at first bleed (day 0; HCV RNA positive and antibody negative), while the Monolisa was reactive 13 days after the first bleed (HCV RNA positive and antibody positive). For DPS eluates, the Murex was only reactive for the 1:5 dilutions at days 7, 13, 16, and 26. However, the Murex failed to be reactive for any of the higher DPS eluate dilutions (1:10-1:100). In contrast, the Monolisa was reactive for antibody positive DPS dilutions of 1:5-1:15 for the bleeds between days 16-35. It was also reactive for the higher dilutions (1:50 and 1:100) at later time points (between days 28-35).

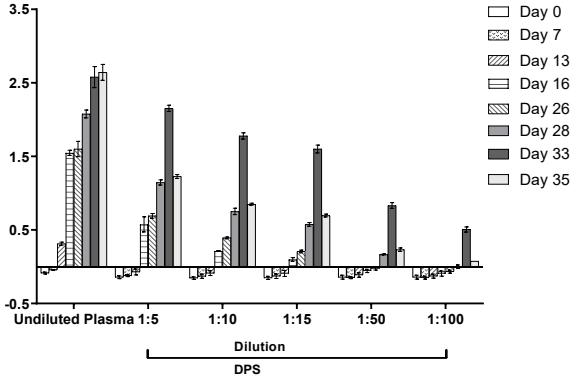
For HCV subtype 2b plasma reference samples, the Murex became reactive 20 days after the first bleed (HCV RNA positive and antibody negative) while the Monolisa was reactive 85 days after the first bleed (HCV RNA negative and antibody positive). For dilutions of DPS eluates, the Murex was reactive for the 1:5 on the days 85, 138-152 and for 1:10 at days 146-152. In contrast, the Monolisa was reactive to all DPS eluate dilutions starting from day 85 until day 152 (HCV antibody positive) (Fig. 12B).

A. HCV subtype 1a

Murex Ag/Ab HCV combination



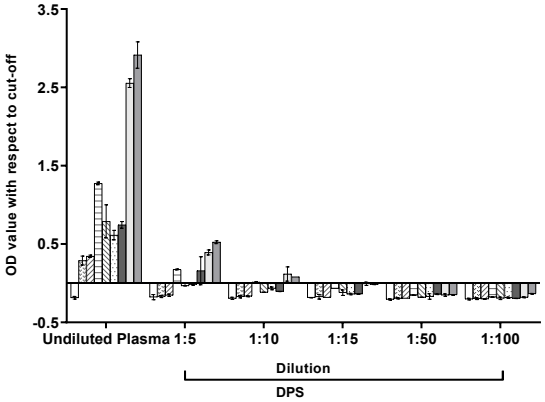
Monolisa HCV Ag/Ab ULTRA



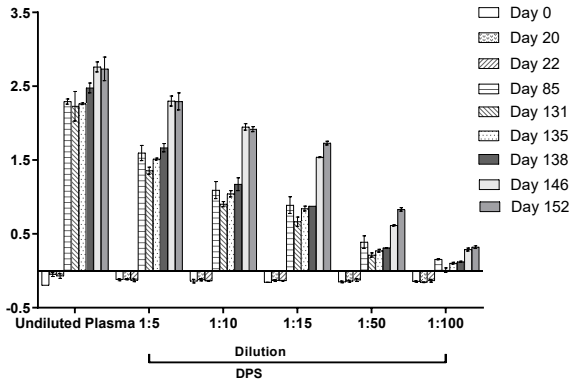
HCV subtype 1a	Days since 1st bleed								
	0	7	13	16	20	26	28	33	35
HCV RNA	+	+	+	+	+	+	+	-	-
HCV Antibody	-	-	+	+	+	+	+	+	+

B. HCV subtype 2b

Murex Ag/Ab HCV combination



Monolisa HCVAg/Ab ULTRA



HCV subtype 2b	Days since 1st bleed								
	0	20	22	85	131	135	138	146	152
HCV RNA	-	+	+	-	-	-	-	-	-
HCV Antibody	-	-	-	+	+	+	+	+	+

Fig. 12: Evaluation of Murex and Monolisa Ag/Ab combination assays for DSS in different dilutions for HCV subtype 1a (A) and subtype 2b (B) seroconversion panels.

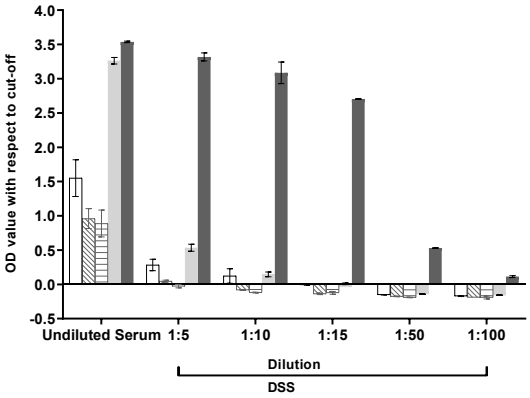
For a panel of HCV subtype 1b serum reference samples, the Murex and Monolisa were reactive for all the days of bleed with undiluted sample (HCV RNA and antibody positive). Additionally, the Monolisa was reactive for all DSS eluate dilutions across the bleed. The Murex however, was only positive for all dilutions with the latest bleed (day 168) (Fig. 13A).

A similar result was also obtained with a panel of HCV subtype 3a HCV RNA and antibody positive serum reference samples. The Murex and Monolisa were reactive at all time points of bleed with undiluted eluates and the Monolisa also for all dilutions tested. The Murex progressively lost its reactivity according to recency and dilution (Fig. 13B).

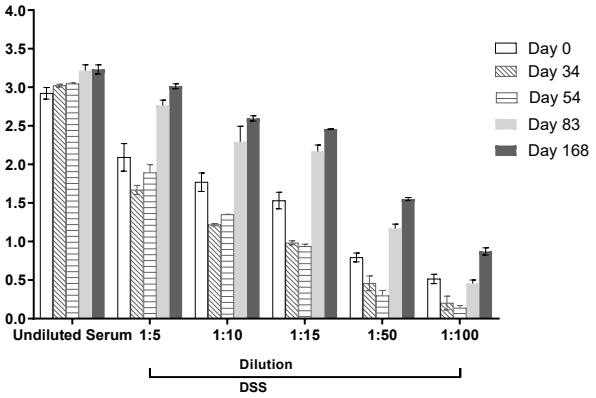
Using a panel of HCV subtype 4d serum samples, the Murex and Monolisa performed akin to the subtype 1b panel (Fig. 13C).

A. HCV Subtype 1b

Murex Ag/Ab HCV combination



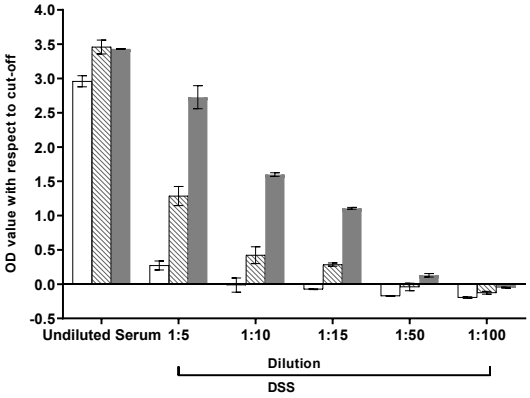
Monolisa HCVAg/Ab ULTRA



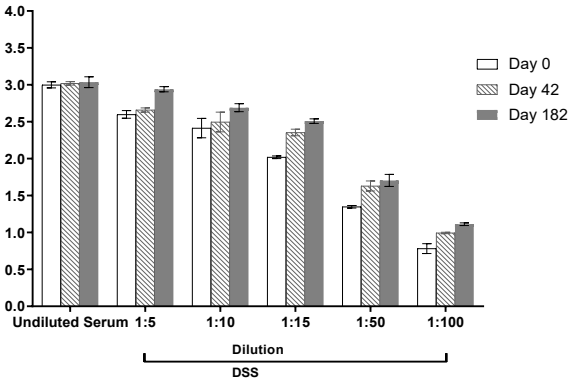
HCV subtype 1b	Days since 1st bleed				
	0	34	54	83	168
HCV RNA	+	+	+	+	+
HCV Antibody	+	+	+	+	+

B. HCV Subtype 3a

Murex Ag/Ab HCV combination



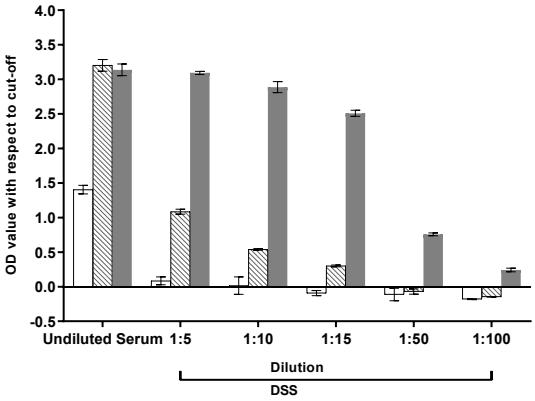
Monolisa HCVAg/Ab ULTRA



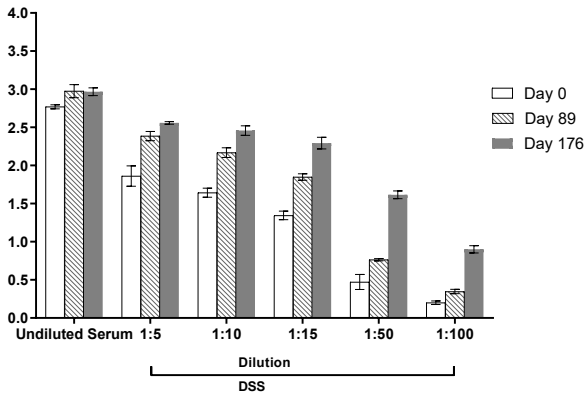
HCV subtype 3a	Days since 1st bleed		
	0	42	182
HCV RNA	+	+	+
HCV Antibody	+	+	+

C. HCV Subtype 4d

Murex Ag/Ab HCV combination



Monolisa HCVAg/Ab ULTRA



HCV subtype 4d	Days since 1st bleed		
	0	89	176
HCV RNA	+	+	+
HCV Antibody	+	+	+

Fig. 13: Evaluation of Murex and Monolisa Ag/Ab combination assay for DSS in different dilutions using HCV subtype 1b (A), subtype 3a (B), and subtype 4d (C) seroconversion panels.

In summary, for the plasma HCV seroconversion samples which were used as a reference for DSS eluates, the Murex became reactive earlier for antigen-positive bleeds. However, for the HCV antibody-positive eluates and dilutions thereof, the Monolisa demonstrated a superior sensitivity.

In order to calculate the percentage of correct HCV positive test results, both assays were also used to screen clinical DSS eluates (n=1,102) obtained from newly diagnosed HIV-infected patients. Of these 123 (11.2%) and 105 (9.5%) DSS samples were found reactive by the Murex and Monolisa tests, respectively. Of those, 87 samples were found reactive in both assays (Table 1) representing 87/123 (70.7%) of the Murex positives and 87/105 (82.9%) of the Monolisa positives. Next, the ELISA reactive samples were screened by RT-qPCR to identify viremic cases. RNA negative reactive samples (presumed resolved infections) were subsequently analyzed by Western blot. Presence of HCV RNA or antibodies was confirmed for 77.2% (n=95/123) of the Murex reactive samples and for 98.1% (n=103/105) of the Monolisa reactive samples. For the Murex, the proportion of false reactive results was therefore higher compared to the Monolisa results (22.8% vs 1.9%) (Table 23).

Of the 64 reactive RT-qPCR-positive samples detected by the Murex, 54 were above the threshold of 1,000 IU/ml of HCV RNA (EASL, 2018) and 10 were below. Of the 65 Monolisa-reactive samples only 5 were below 1,000 IU/ml of HCV RNA. This result is in line with our findings on the seroconverter panels indicating a higher sensitivity of the Murex assay for the detection of the HCV antigen.

Table 23: Comparison of the performance of Murex and Monolisa in screening HCV coinfection in DSS of newly diagnosed HIV cases (n=1,102).

Test system	ELISA reactive	RT-qPCR positive	RT-qPCR negative and Western blot positive	Confirmed ELISA results	
Murex	123/1102 (11.2%)	64/123 (52%)	31/59 (52.5%)	95/123 (77.2%)	P<0.0001
Monolisa	105/1102 (9.5%)	65/105 (61.9%)	38/40 (95%)	103/105 (98.1%)	P<0.0001
Both assays	87/1102 (7.9%)	58/87 (66.7%)	29/29 (100%)	87/87 (100%)	

4.2 RESULTS (PART II)

Monitoring recency of infection helps to identify current transmission in vulnerable populations for effective disease control. In this study, an in-house avidity based HCV recency assay is established based on the Monolisa Anti-HCV PLUS Version 3 ELISA kit for use of DS/PS in order to distinguish recent and long-term infections.

A first panel of DS/PS (n=218; genotype 1 n=170 and non-genotype 1 n=48) consisting of primary and at least one follow up sample was used to analyze the temporal changes of the AI over time. Sub-panels of longitudinal DS/PS (n=66) and acute cases (<26 weeks; n=34) were taken to calculate the MDRI and the FLTR, respectively. A second panel of DS/PS >104 weeks (n=132) and a third panel of DS/PS prepared from resolved infections (≥180 days since last positive; n=32) were used to calculate the FRR.

4.2.1 Clinical characteristics of the study participants

The relevant characteristics of the specimens included into the study are shown in Table 1. Among the 218 DS/PS tested, the majority was from infections with HCV genotype 1 (77.9%). The median age of the study participants was 57 years (95% CI: 53-58).

Table 24: Characteristics of study specimens

	No. of samples	%	No. of individuals	%
Sex				
Male	150	68.8	43	67.2
Female	64	29.4	20	31.3
Unknown	4	1.8	1	1.5
HCV genotype				
Genotype 1	170	77.9	51	79.7
Genotype 3	18	8.3	6	9.4
Genotype 4	30	13.8	7	10.9
Estimated duration of infection				
<26 weeks	34	15.6	18	19.4
26-52 weeks	28	12.8	16	17.2
53-104 weeks	24	11	18	19.4
>104 weeks	132	60.6	41	44
HIV Status				
Positive	75	34.4	21	32.8
Negative	143	65.6	43	67.2
Evaluation panel				
Panel 1 (Longitudinal sample set)	218	100	64	100
Sub panel a (for MDRI)	66	30.3	18	28.1
Sub panel b (for FLTR)	34	15.6	18	28.1
Panel 2 (for FRR)	132	60.6	41	64.1
Panel 3 (Resolved infection)	32	100	12	100

The increase of antibody avidity with time for genotype 1 and non-genotype 1 infections has been determined. Higher AI values were generally obtained for genotype 1 compared to non-genotype 1 samples (Fig.14).

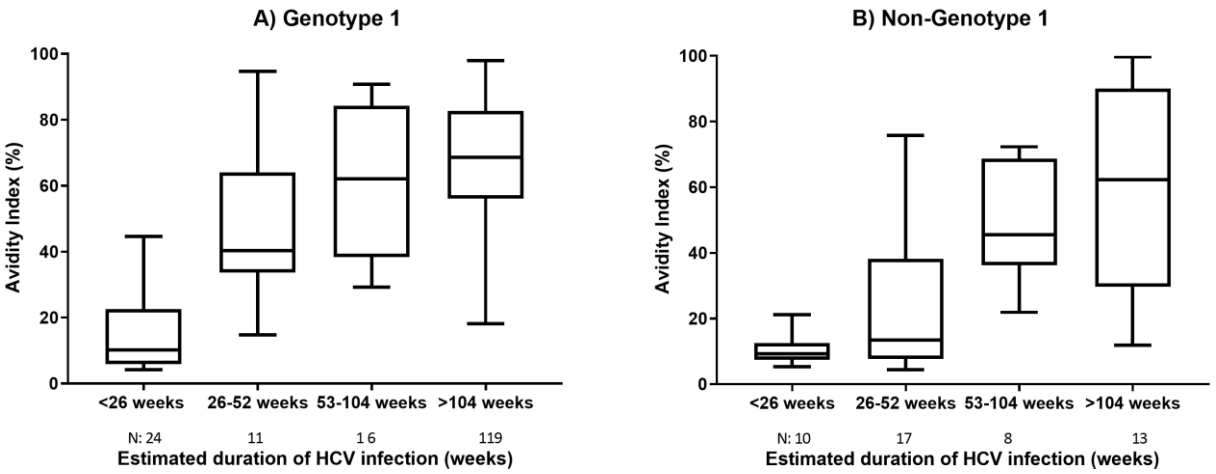


Fig. 14: Differential increase in HCV IgG antibody avidity over time for genotype 1 (A) and non-genotype 1(B) infections. An AI value was determined for 218 samples (from 64 individuals) with HCV RNA ≥250 IU/ml. The median AI increases progressively with duration of infection for both genotype 1 and non-genotype 1 infections. n = number of samples

4.2.2 Sensitivity, specificity and MDRI

The longitudinal dataset (subpanel 1a; n=66) allowed estimating the probability for a recent infection by a mixed-effects logistic regression model for all genotypes with durations of infection of up to 4 years (Fig. 15). The sensitivity and specificity calculated by the model are only used to find the optimal AI and MDRI at AI cut-off values ranging from 20-45%. As shown in table 25, the sensitivity has increased from 72.4% at an AI cut-off of 20%, to 85.7% at an AI cut-off of 40%, while the specificity has increased from 72.9% at an AI cut-off of 20%, to 88.2% at an AI cut-off of 40%. However, at an AI cut-off to 45%, both sensitivity and specificity have shown a decrease to 80.3% and 70%, respectively. The optimal AI of 40% resulted in an MDRI of 364 days (95% CI: 223-485) for all genotypes with an accuracy (number of correctly classified samples) of 86.9%.

Table 25: Sensitivity, specificity, accuracy and Mean Duration of Recent Infection (MDRI) according to selected cut-off avidity index (AI) values calculated for the longitudinal dataset (subpanel 1; n=66).

AI cut-off (%)	Sensitivity (%)	Specificity (%)	Accuracy (%)	MDRI (days)	(95% CI)
All genotypes					
20	72.4	72.9	72.6	233	(149-449)
25	78.3	79.3	78.8	264	(179-634)
30	81.5	82.1	81.8	268	(183-695)
35	82.2	80.9	81.5	325	(228-445)
40	85.7	88.2	86.9	364	(223-485)
45	80.3	70	75.1	517	(16-643)

A mixed-effects logistic regression was used with the duration after infection and its square as fixed effects and the identifier of the patient as random effect. Since only few samples from patients with duration of infection longer than 2 years could be included, the confidence intervals are large for those durations (Fig. 15).

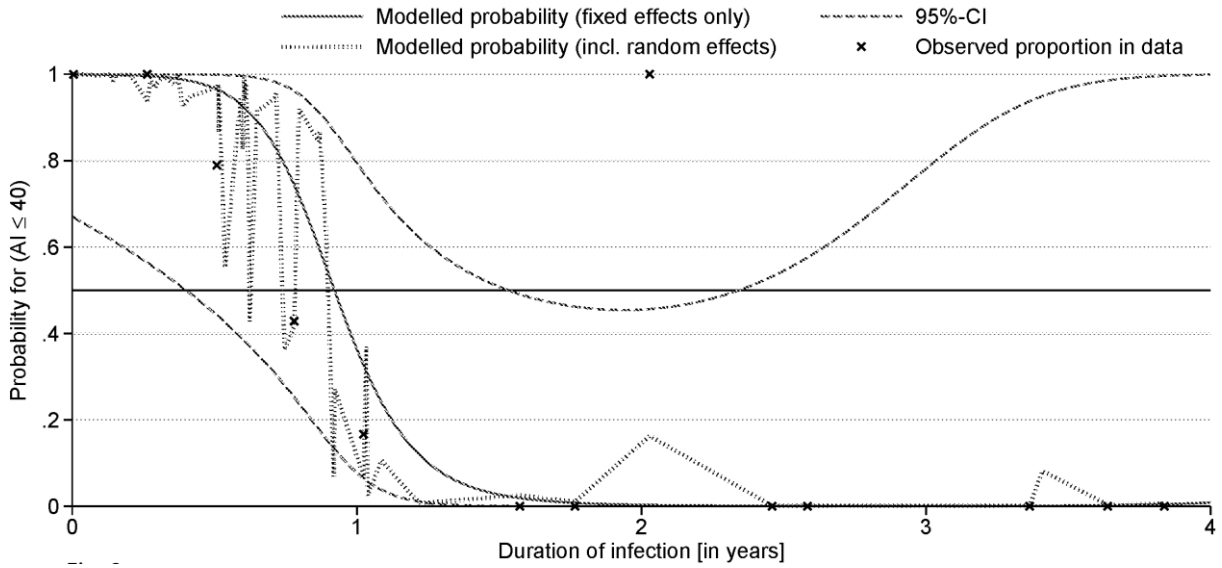


Fig. 15: Estimated probability for a positive avidity assay result as a function of the duration of the HCV infection estimated by the mixed effect logistic regression model.

The FRR calculated using panel 2 (estimated time since infection of >104 weeks) for the optimal AI cut-off 40% calculated from samples with a long-term infection was 13.6%

(95% CI: 8.3-20.7) for all genotypes, 11.7% (95% CI: 6.6-19) for genotype 1, and 30.6% (95% CI: 9.1-61.4) for non-genotype 1 HCV infections. At the same AI cut-off value, the FLTR calculated using panel 1b (estimated time since infection of <26 weeks) was found to be 5.9 (0.7-19.7) for all genotypes, 8.3 (1-27) for genotype 1, and 0% (-) for non-genotype 1 HCV infections (Table 26).

Table 26: Estimated `False Recent Rate´ (FRR) and `False Long-term Rate (FLTR)´ calculated from the `long-term´ (> 104 weeks) and `recent´(< 26 weeks) infection panel according to the AI cut-off and genotype.

	Avidity Index (%)					
	20	**25**	**30**	**35**	**40**	**45**
FRR %						
(95% CI)						
All genotypes	2.2	6.8	9.1	11.4	13.6	16.7
(n=132)	(0.5-6.5)	(3.2-12.5)	(4.8-15.3)	(6.5-18)	(8.3-20.7)	(10.7-24.1)
Genotype 1	0.8	5.8	7.5	9.2	11.7	14.3
(n=119)	(0-4.6)	(2.4-11.7)	(3.5-13.9)	(4.7-15.9)	(6.6-19)	(8.5-21.9)
Non-genotype1	15.4	15.4	23.1	30.6	30.6	38.5
(n=13)	(1.9-45.4)	(1.9-45.4)	(5-53.8)	(9.1-61.4)	(9.1-61.4)	(13.9-68.4)
FLTR %						
(95% CI)						
All genotypes	26.5	14.7	11.8	8.8	5.9	0
(n=34)	(12.9-44.4)	(5-31.1)	(3.3-27.5)	(1.9-23.7)	(0.7-19.7)	(-)
Genotype 1	33.3	20.8	16.7	12.5	8.3	0
(n=24)	(15.6-55.3)	(7.1-42.2)	(4.7-37.4)	(2.7-32.4)	(1-27)	(-)
Non-genotype1	10	0	0	0	0	0
(n=10)	(0.3-44.5)	(-)	(-)	(-)	(-)	(-)

4.2.3 AI value for resolved infection

Samples with a resolved infection were used in order to evaluate the performance of the assay in anti-HCV positive and HCV RNA negative cases. All the samples which had a minimum follow-up of 180 days since the estimated date of infection and before clearing the infection have a decline in the AI overtime. The mean AI values before (HCV RNA positive test) and after (HCV RNA negative test) clearing the infection were 62.4% and 43.5%, respectively.

Furthermore, of the 32 DS/PS specimen tested as resolved infection, 19 had AI values below the optimal AI cut-off (40%) resulting in a FRR of 53.1% (95% CI: 35.8-70.4) (Fig. 16).

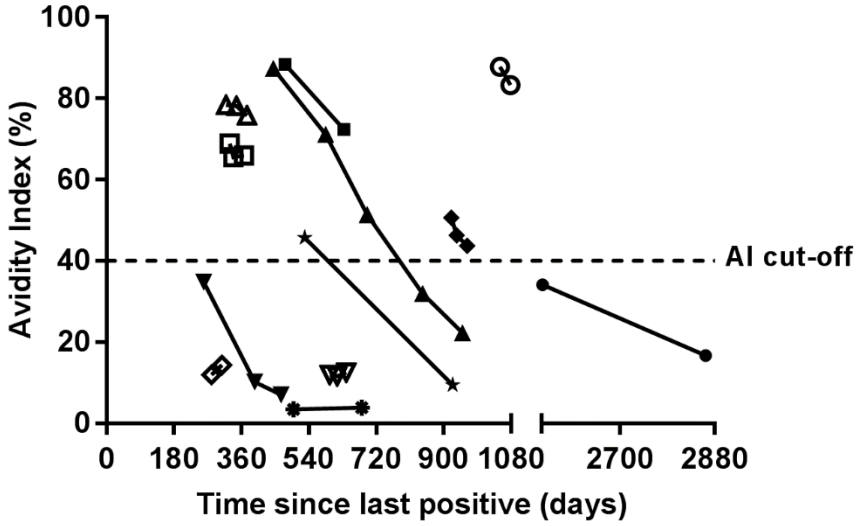


Fig. 16: Changes in anti-HCV IgG AI values over time for individuals with resolved HCV infection. The observation period (2,863 days) is expressed as a minimum of 180 days before clearing the infection. The AI cut-off at 40% is indicated by a dashed line. Each symbol represents a single individual.

In summary, this assay performs particularly well for genotype 1 reaching a high rate of correct discriminations between infections acquired less than a year before diagnosis and those acquired earlier by applying an AI cut-off of 40%. Due to a rapid decline in avidity post resolution of an HCV infection this assay is not recommended to be used in HCV RNA negative patients.

4.3 RESULTS (PART III)

The objective of this part of the study was to determine the prevalence of HCV, HCV genotype/subtypes and RAS among a subset of new HIV-1 infections diagnosed between 2015 and 2017 in Germany. For this purpose, a total of 6,097 DSS prepared from residual serum of newly HIV-diagnosed cases were sent to the RKI along with the anonymous report. This part of the study aimed to analyze HCV genotypes/subtypes and RAS in the main transmission groups: MSM, PWID and heterosexuals while considering sex and origin of the infected individuals. The total number of DSS tested in this study represents the general newly diagnosed HIV positive population in Germany ($p<0.05$) as shown by an approximation of the analyzed variables to the national statutory report (Fig. 17).

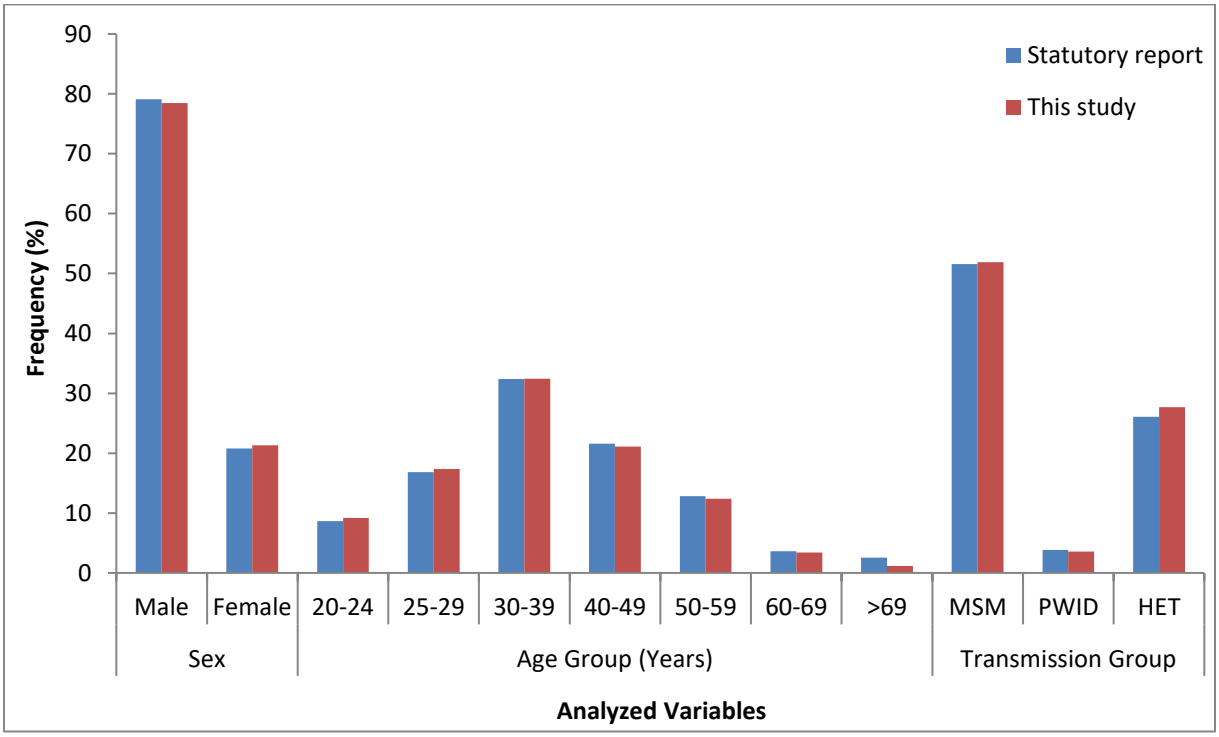


Fig. 17: Comparison of samples analyzed in this study with the national statutory report with regards to some socio-demographic variables.

4.3.1 HCV prevalence

Of the total 6,097 tested samples, 416 (6.8%) were found to be ELISA reactive indicating the possible presence of anti-HCV antibody or HCV antigen in the patient samples. Analysis of these ELISA reactive samples by qRT-PCR for the presence of HCV RNA revealed 256 (4.2%) positive samples showing an active HCV infection. In order to confirm the presence of

HCV specific antibodies among ELISA reactive and HCV-RNA negative samples, a further analysis of available 140 out of 160 samples was performed by Western blot. For the remaining 20 samples, there was no material available to carry out the Western blot analysis. Therefore, of the tested 140 samples, 120 (85.7%) became Western blot positive. Thus, 20 out of the 416 ELISA reactive samples (4.8%) could be identified as false ELISA-positives. The inability to confirm a positive fourth generation HCV-ELISA result by Western blot with an HCV RNA negative sample provides evidence for a false positve ELISA result (Rouet *et al.*, 2015). However, if the whole 160 samples would have been tested instead of only 140, a presumed 23 samples would have been false positives resulting in 393 correct ELISA reactives. This could mean that 3 out of the 20 ELISA reactive and RNA negative samples that could not have been tested would presumably be false positives. Since we do not know which these three samples are and in order to stay with lab results and avoid the possibility of introducing a bias when calculating the total ELISA reactives, only the 20 samples that were negative in the Western blot were removed from the 416 ELISA reactive samples resulting in 396 diagnosed HCV coinfections for subsequent analyses. The proportion of viremic samples is 64.6% (256/396) and the remaining cases (n=140; 35.4%) are resolved HCV coinfections.

Analysis of the samples in each of the diagnosis years showed the highest proportion of HCV seropositive persons (7.3%, n=153/2,087) and active infections (5.4%, n=112/2,087) in the year 2016 [comparison to 2015: 6.9% (n=136/1,980) and 4.2% (n=83/1,980), respectively]. However, in the diagnosis year 2017, there was a significant decline in the proportions of both HCV seropositive persons (5.3%, n=107/2,030; $p<0.01$) and active infections (3%, n=61/2,030; $p<0.001$) (comparison 2016: 7.3% and 5.4%, respectively) (Fig. 18).

A study on samples with HIV new diagnosis from the pre-DAA study (October 2009 to June 2011) by Johanna Riege (information extracted from her Bachelor thesis) (Riege *et al.*, 2019) was taken for comparison with the analysis of this (post-DAA) study (2015-2017). The analysis of the pre-DAA study showed HCV antibody presence in 87 out of the 1,511 samples tested by the same ELISA. Of these, 81 (5.4%) samples could be further assayed using qRT-PCR and 62 (4.1%) samples were tested positive indicating active infections. The proportion of resolved among all infections was 23.5% (Fig. 19).

A comparison of the pre-DAA study with this study showed an increase in the proportion of HCV seropositive patients (5.4%, n=81/1,511 and 6.5%, n=396/6,097, respectively).

However, a similar proportion of active infection was observed in both studies (4.1%, n=62/1,511 and 4.2%, n=256/6,097, respectively) (Fig. 18).

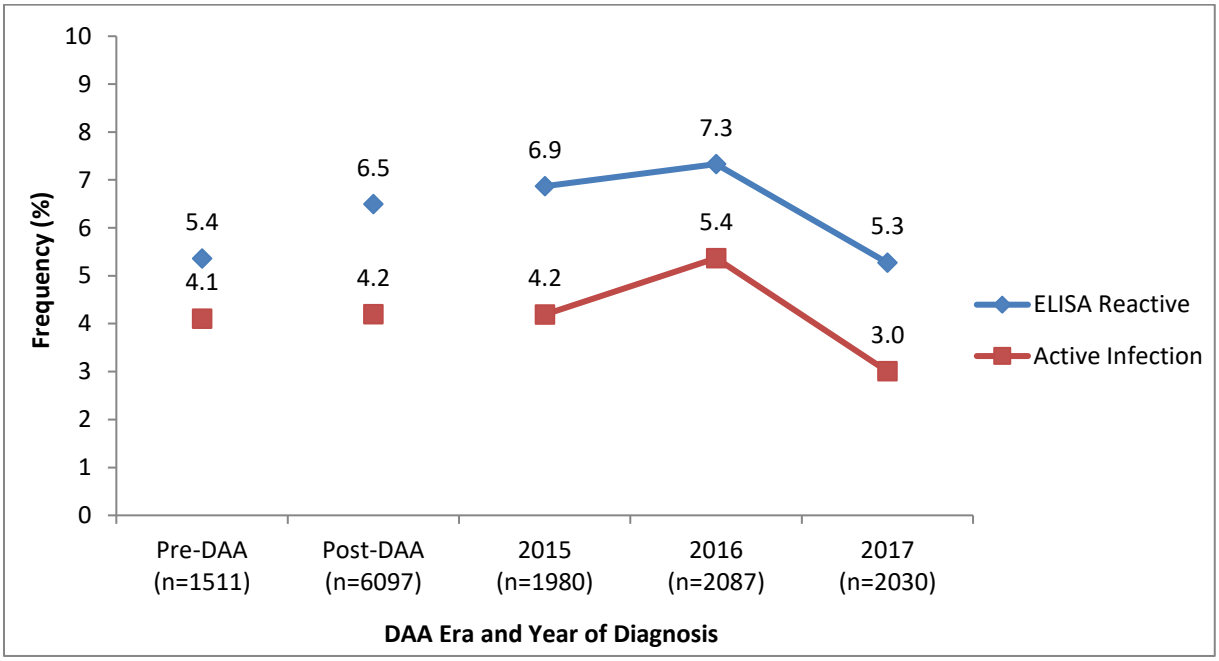


Fig. 18: Proportion of HCV ELISA reactive and active infections among HIV new diagnoses in the pre-DAA (2009-2011) and post-DAA (2015-2017) studies. n = number.

The proportion of resolved infections among the diagnosed HCV coinfections has shown a significant increase ($p<0.01$) from the lowest proportion in the diagnosis year 2016 (26.8%; n=41/153) to a highest proportion in the year 2017 (43%; n=46/107). The increase in the overall proportion of resolved infections was also significantly higher ($p<0.05$) in this study than the pre-DAA study (23.5%; n=19/81 vs 35.4%; n=140/396) (Fig. 19).

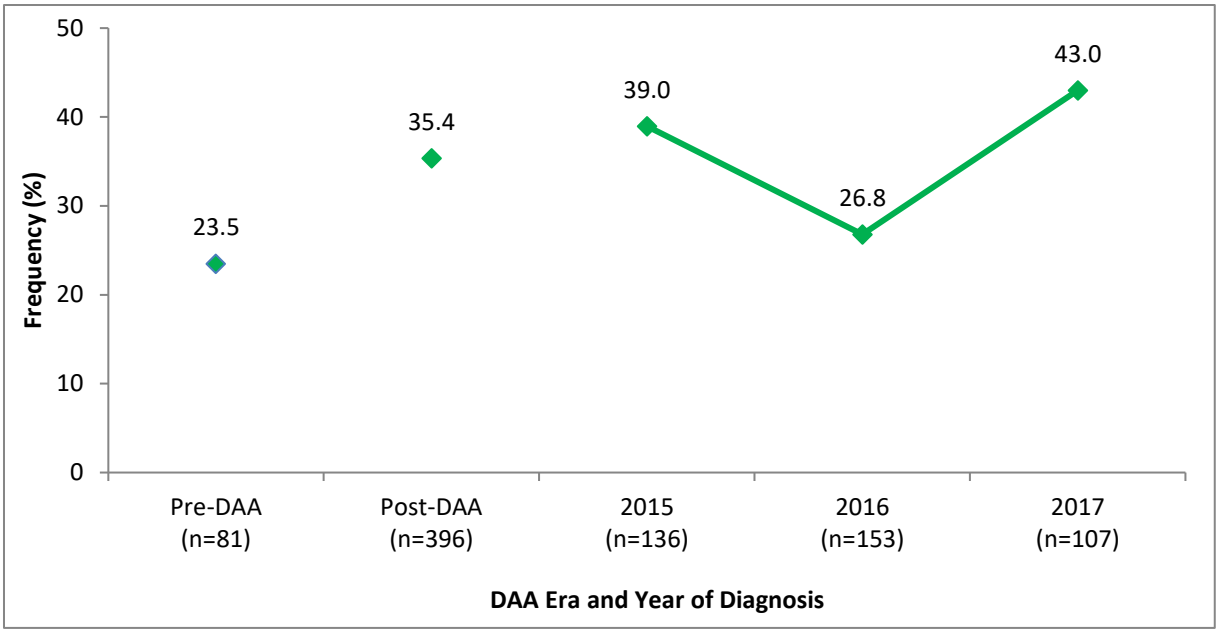


Fig. 19: Proportion of resolved among all HCV resolved coinfections in HIV new diagnoses in the pre-DAA (2009-2011) and post-DAA (2015-2017) studies.

4.3.2 Recent and longstanding HCV infections

To estimate the trajectory of HCV infections in the country, an analysis of recent and long-term infections was made using the established HCV recency assay. Accordingly, the proportion of HCV long-term infections was relatively higher in all diagnoses years (2015: 72%; 2016: 70%; 2017: 63%) (Fig. 20).

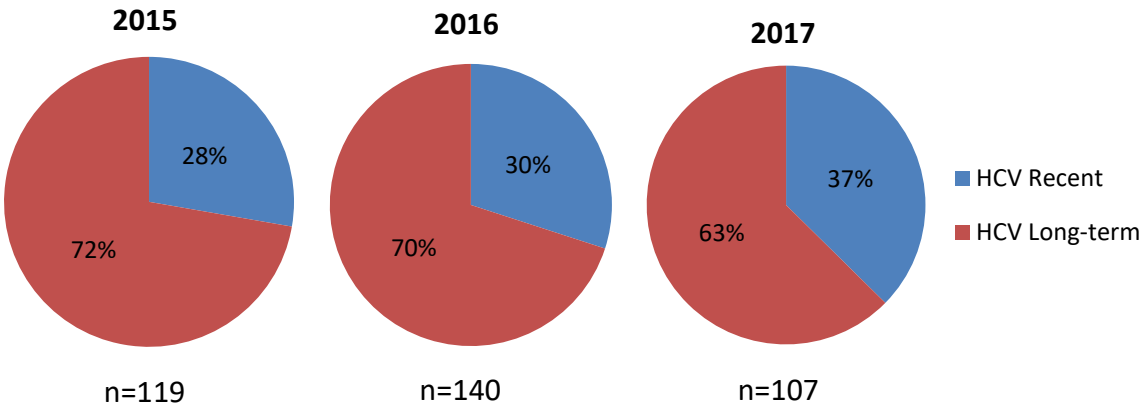


Fig. 20: Distribution of recent and long-term HCV infections among HIV new diagnoses in the years 2015-2017.

To further estimate the trends of HCV/HIV coinfection, an analysis of recent and long-term HCV/HIV coinfections was made using the established HCV recency assay. As a result, the proportion of long-term HCV infections in long-term HIV infections was higher in all diagnoses years (2015: 54%; 2016: 39%; 2017: 39%). However, the proportion of long-term HCV infections in long-term HIV infections has shown a significant decline ($p<0.01$) between the diagnoses years 2015 and 2016. On the other hand, during the same period, a significant increase ($p<0.01$) in the proportion of HCV long-term infections in HIV recent infections was observed. A lower proportion of HCV recent infections in HIV recent infections were also observed in all diagnoses years (2015: 7%; 2016: 5%; 2017: 11%) (Fig. 21).

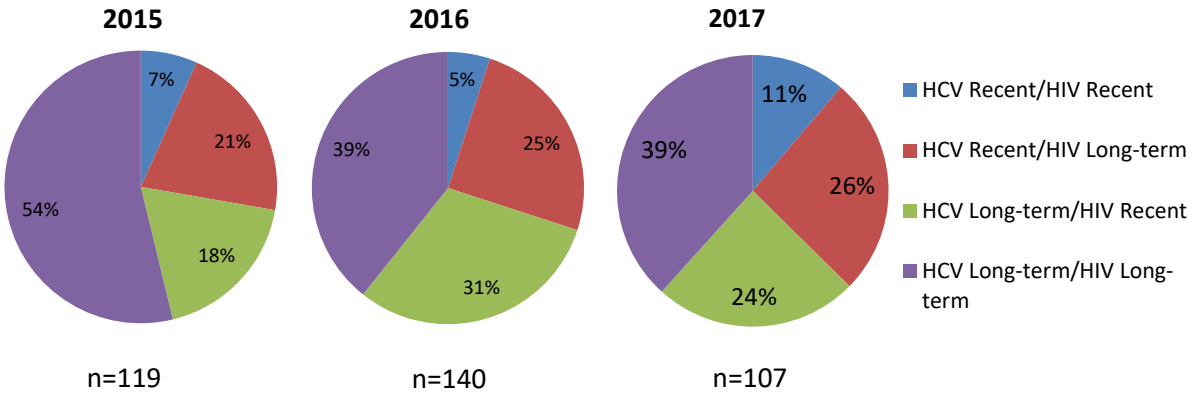


Fig. 21: Distribution of recent and long-term HCV infections in recent and long-term HIV infections in the year of diagnoses 2015-2017.

4.3.3 HCV infection in different transmission groups

In the samples analyzed for the diagnoses years 2015-2017, the main transmission groups were MSM (51.9%, n=3,164/6,097) followed by heterosexuals (27.7%, n=1,686/6,097) and PWIDs (3.5%, n=216/6,097). Although PWIDs accounted for only a small proportion of the study participants (3.5%), this group has the highest contribution of HCV seropositive samples (42.4%, n=168/396) as well as active (45.7%, n=117/256) and resolved (36.4%, n=51/140) HCV coinfections. On the other hand, in comparison with the other transmission groups, the PWIDs have the lowest contribution of HCV seronegative cases (0.8%, n=45/5681) (Fig. 22). Moreover, PWIDs are more likely to have HCV coinfection than the other transmission groups (OR=79.6, $p<0.0001$) (Table 27). The MSM group constitutes 19.9% (n=79/396) of the tested HCV seropositive and the majority of the HCV seronegative cases (54.1%, n=3,074/5,681) as well as 19% (n=48/256) of the active and 22.1% (n=31/140)

of the resolved HCV coinfections. The heterosexuals group constitutes 18.7% (n=74/396) of the HCV seropositive and 28.5% (n=1,608/5,681) of the seronegative cases. This group also includes 15% (n=38/256) and 25.7% (n=36/140) of the total active and resolved HCV coinfections, respectively. It is also important to also note that PWIDs, in comparison to other transmission groups, contribute to the majority of HCV positive, resolved (except for the diagnosis year 2015 wherein heterosexuals contribute the highest, 37.7%) and active HCV infections in the pre- and post-DAA studies and throughout the entire study period (2015-2017) (Fig.22).

In comparison with the pre-DAA study, in this study, there was a significantly higher proportion of DSS (17%, n=264/1,511 vs 28%, n=1,686/6,097; P<0.0001) and HCV seronegative cases obtained from heterosexuals (18%, n=255/1,422 vs 28%, n=1,608/5,681; P<0.0001) and a significantly lower proportion of HCV seronegative cases obtained from MSM (62%, n=875/1,422 vs 54%, n=3,074/5,681; P<0.0001) (Fig. 22).

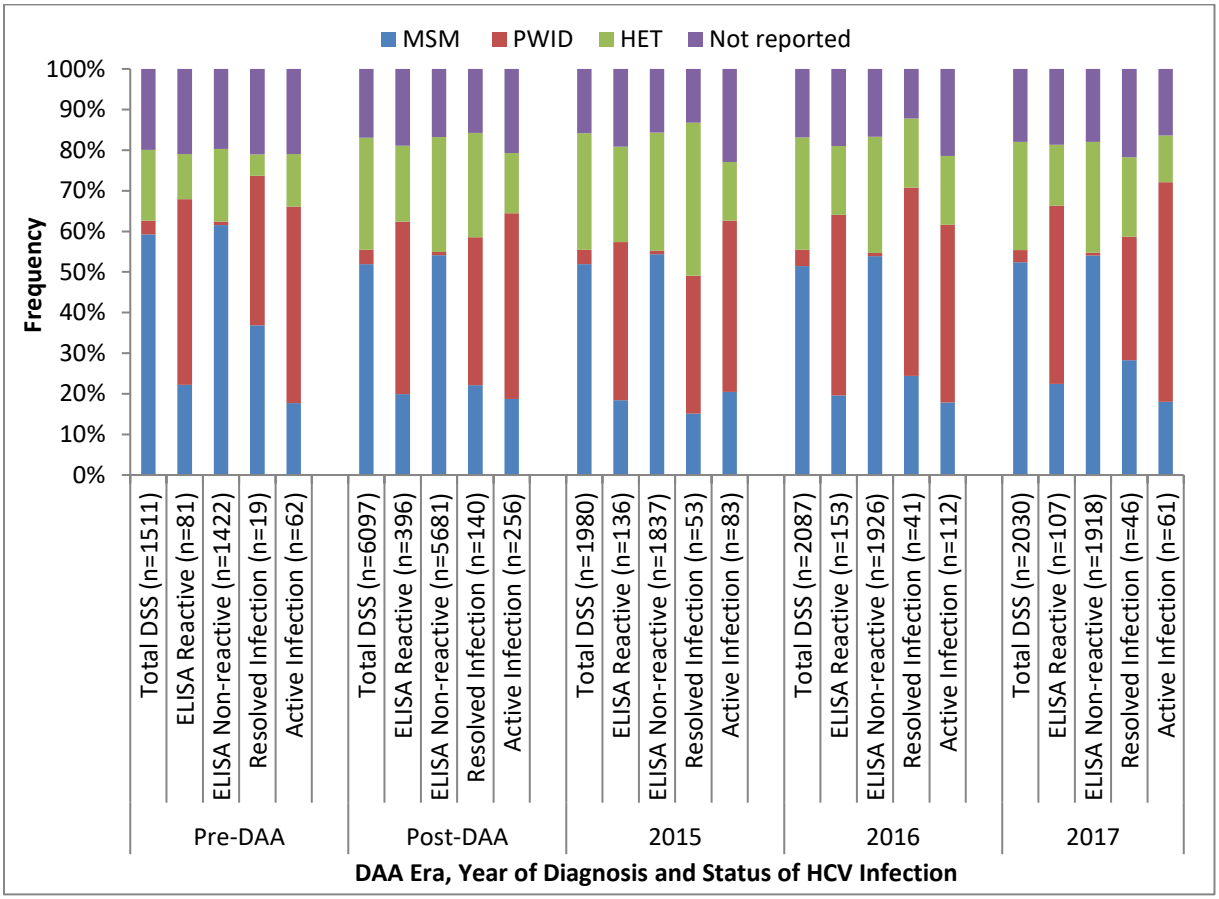


Fig. 22: Proportion of transmission groups and status of HCV infection among HIV new diagnoses in the pre-DAA (2009-2011) and post-DAA (2015-2017) studies. MSM = Men who have Sex with Men, PWID = People Who Inject Drugs, HET = Heterosexuals.

Table 27: Odds ratio for HCV infection in transmission groups of HIV new diagnosis years 2015-2017.

Transmission Group	OR	95% CI	p-value
Men who have sex with men	0.12	0.09-0.16	p<0.0001
People who inject drugs	79.6	55.6-113.8	p<0.0001
Heterosexuals	0.42	0.32-0.55	p<0.0001

A further look at the proportion of HCV coinfections in each transmission group revealed PWIDs to have the highest proportion (77.8%, n=168/216) followed by heterosexuals (4.4%, n=74/1,686) and MSM (2.5%, n=79/3,164). The proportion of HCV coinfection remained similar throughout the diagnoses years (2015-2017) in PWIDs (between 75.7 and 81%) and MSM (between 2.4 and 2.8%). In heterosexuals, however, there was a significant decline (p<0.05) between the diagnoses years 2015 and 2017 (5.6%, n=32/568 vs 3%, n=16/540). A comparison of the pre-DAA study with this study also showed a similar proportion in MSM (2.1% and 2.5%, respectively), PWIDs (76.5% and 77.8%, respectively) and a slight increase (p=0.45) in heterosexuals (3.4% and 4.4%, respectively) (Fig. 23).

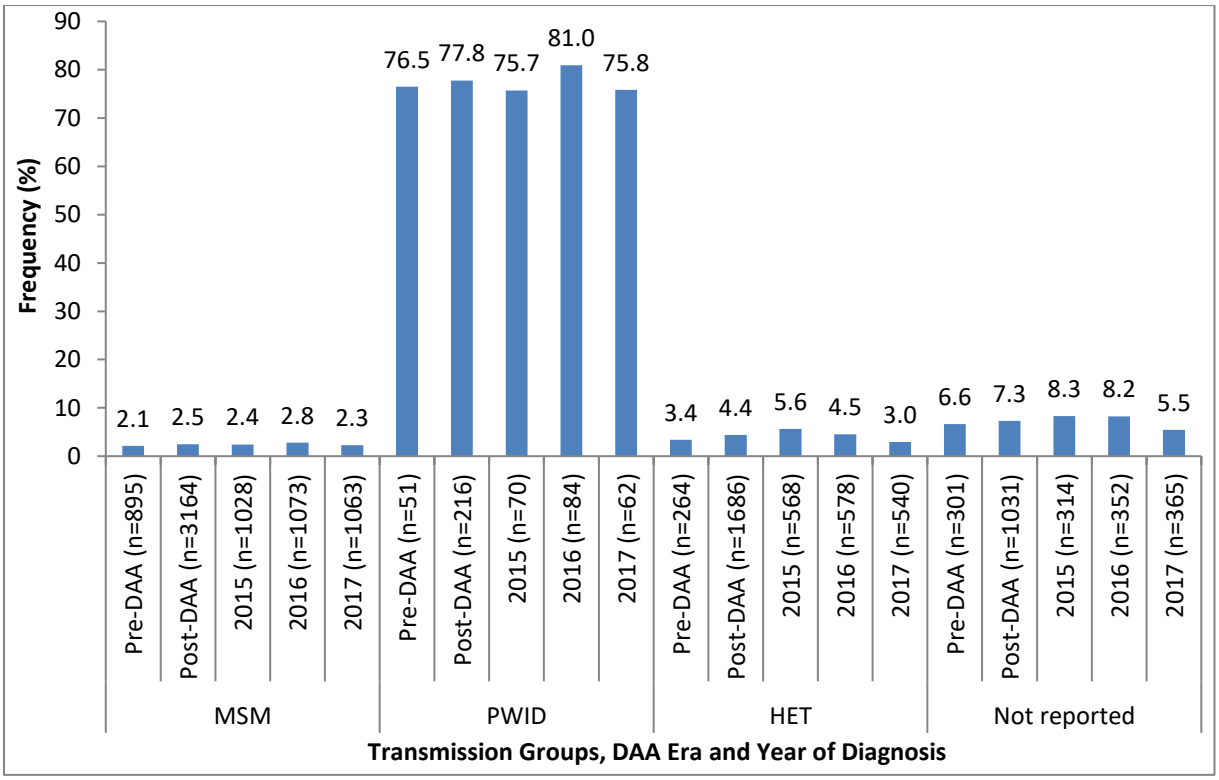


Fig. 23: Proportion of HCV infections belonging to a certain transmission group in the pre-DAA (2009-2011) and post-DAA (2015-2017) studies.

Analysis of resolved HCV coinfections among ELISA reactive individuals in each of the transmission groups showed the highest proportion in heterosexuals (53.7%, n=36/67) followed by MSM (42.5%, n=31/73) and PWIDs (31.3%, n=51/163). A further comparison of resolved infections through the diagnoses years (2015, 2016 and 2017) showed a similar proportion in MSM between the years 2015 and 2016 (36.4%; n=8/22 and 35.7%; n=10/28, respectively) and an increase (p=0.14) in the year 2017 (56.5%, n=13/23). For PWIDs and heterosexuals, compared to the year 2015, a decrease was observed in the year 2016 (36% vs 28.4%; p=0.38 and 76.9% vs 28%; p<0.001, respectively) followed by an increase in the year 2017 (30.4%; p=0.81 and 56.3%; p=0.07, respectively) (Fig. 24).

Compared with the pre-DAA study, the proportion of resolved HCV coinfections in the post-DAA study showed an increase in PWIDs (18.9%, n=7/37 vs 31.3%, n=51/163; p=0.13), heterosexuals (11.1%, n=1/9 vs 53.7%, n=36/67; p<0.05) and a slight increase in MSM (38.9%, n=7/18 vs 42.5%, n=31/73; p=0.78) (Fig. 24).

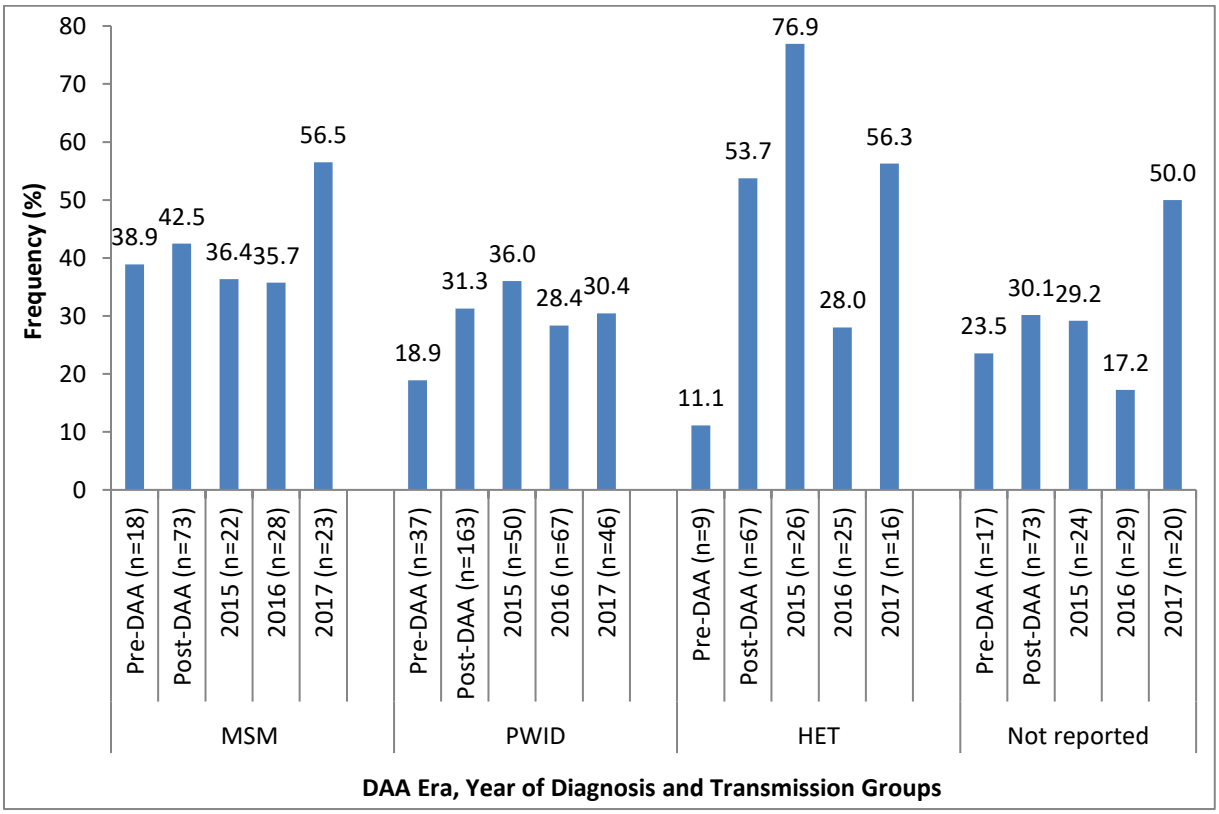


Fig. 24: Proportion of resolved HCV coinfections among HIV new diagnoses in different transmission groups of the pre-DAA (2009-2011) and post-DAA (2015-2016) studies.

Based on the data obtained from the established recency assay, a further analysis on the extent of recent and long-term HCV/HIV coinfections was performed for the different transmission groups. Thus, a higher proportion of HCV long-term in HIV long-term cases were observed in MSM (37%, n=27/73), PWIDs (46.5%, n=72/155) and heterosexuals (45.5%, n=30/66). There were significant associations of PWIDs and heterosexuals with HCV long-term in HIV recent (35.5%; $p<0.01$) and HCV recent in HIV long-term (33.3%; $p<0.05$) cases, respectively (Fig. 25).

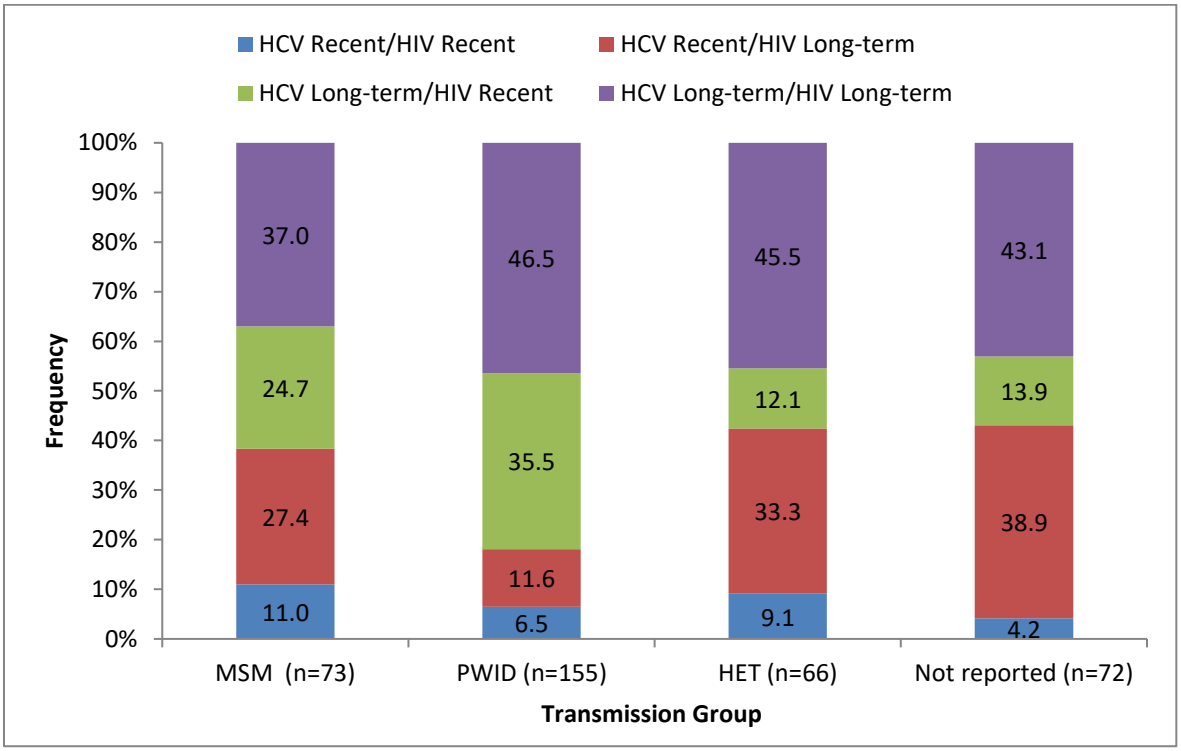


Fig. 25: Distribution of recent and long-term HIV infections in recent and long-term HCV infections in different transmission groups during the diagnoses years 2015-2017.

Analysis on the trends of recent and long-term HCV/HIV infections was made for each of the main transmission groups. In the MSM, an increase in the proportions of HCV recent in HIV recent cases was observed in the years of diagnosis 2015 (0%, n=0/23), 2016 (11.5%, n=3/26) and 2017 (20.8%, n=5/24). The increase in MSM was significantly higher ($p<0.05$) between the diagnoses years 2015 and 2017. In the heterosexuals, although not significant ($p>0.05$), an increasing proportion of HCV long-term infections in HIV recent infections can be seen in the years 2015 (3.8%, n=1/26), 2016 (16.7%, n=4/24) and 2017 (18.8%, n=3/16) (Fig. 26).

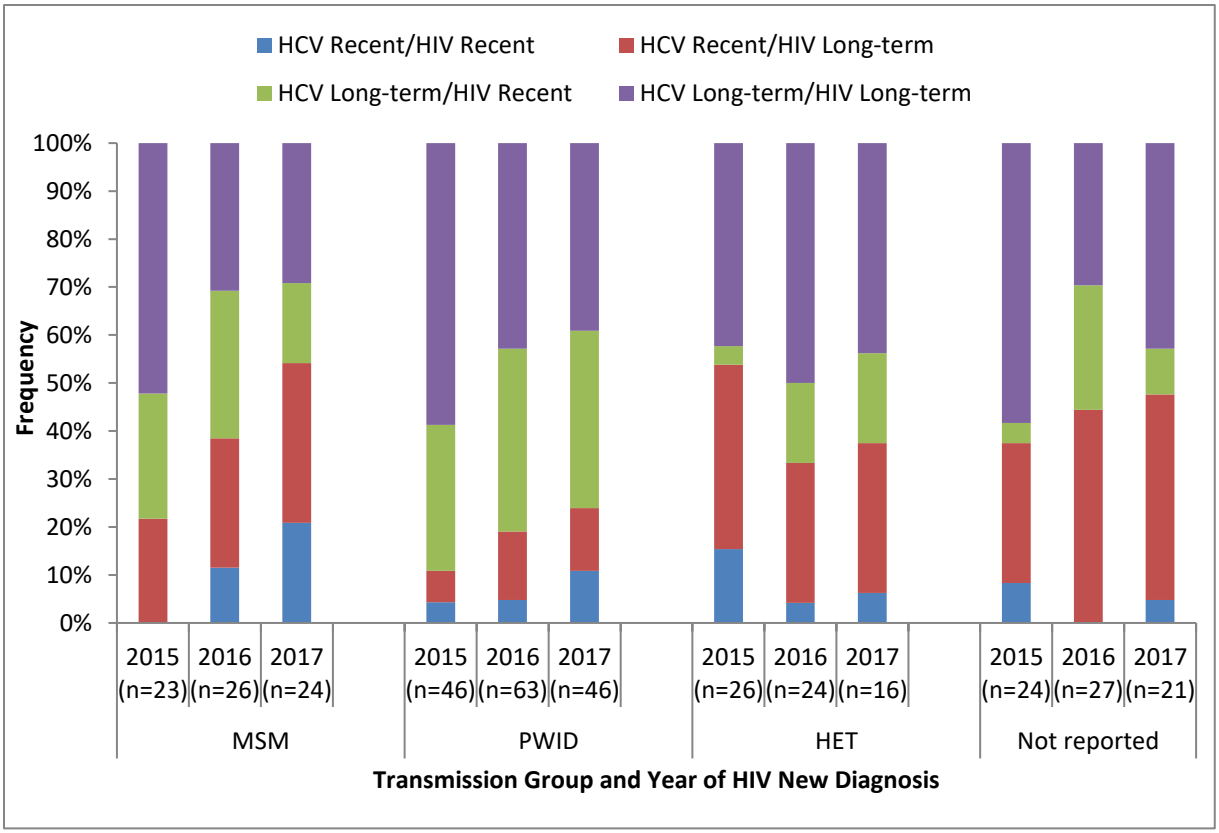


Fig. 26: Overview of the proportions of recent and long-term HIV infections in recent and long-term HCV infections in different transmission groups for the HIV diagnoses years 2015-2017.

4.3.4 HCV infection by sex groups

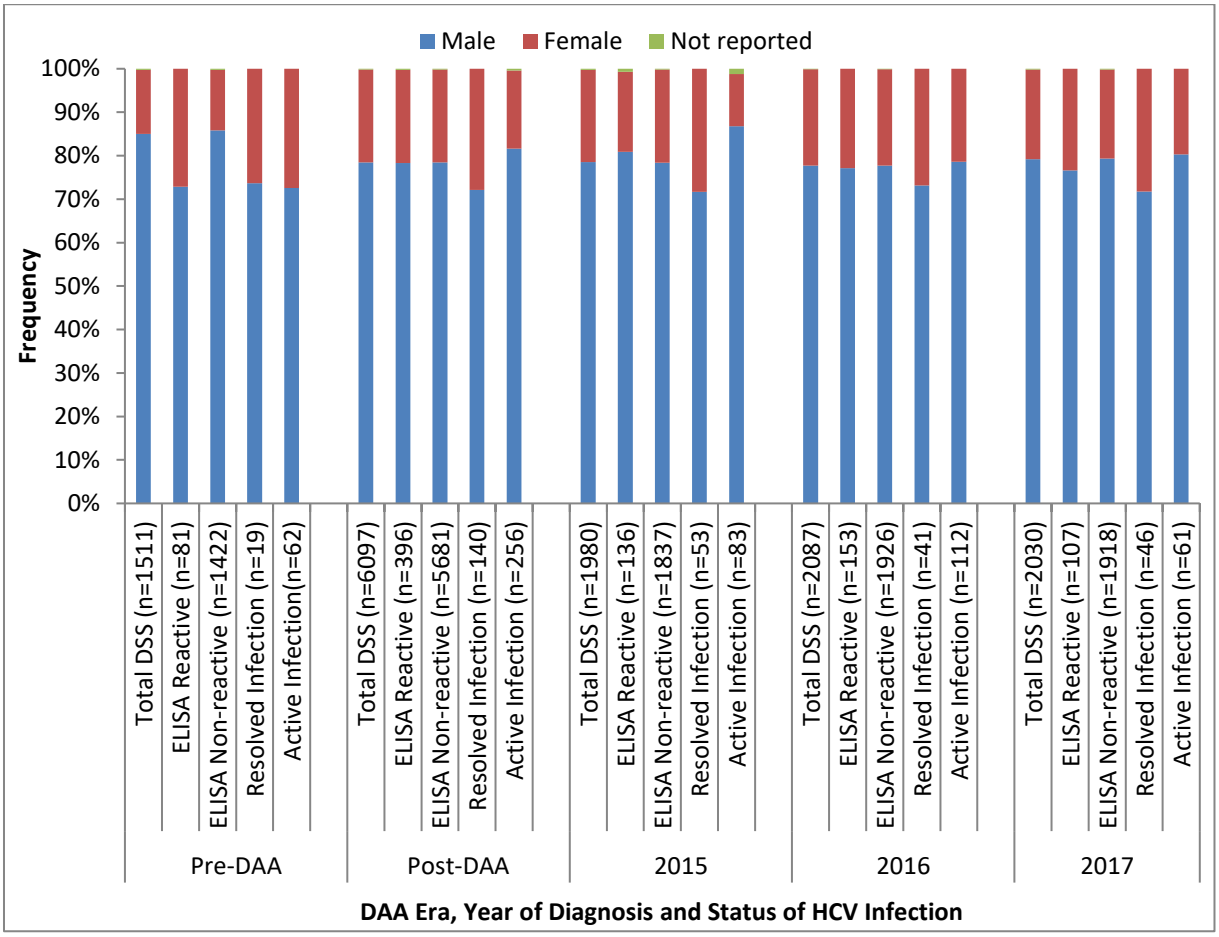
To assess whether there is a difference in the prevalence of HCV coinfection in males and females, an analysis of HCV infection in relation to both sex groups was made for the current and pre-DAA studies. Among the overall samples tested for the diagnoses years 2015-2017, males account for the highest proportion of DSS tested (78.5%, n=4,785/6,097; p<0.0001), HCV seropositive (78.3%, n=310/396; P<0.0001) and seronegative individuals (78.5%, n=4,459/5,681; P<0.0001) as well as active (81.6%, n=209/256; P<0.0001) and resolved HCV coinfections (72.1%, n=101/140; P<0.0001). This has also been reflected in each of the diagnoses years (2015-2017) where males consistently dominate the proportion of HCV seropositive (80.9%; n=110/136, 77.1%; n=118/153 and 76.6%; n=82/107, respectively) and seronegative (78.4%; n=1,440/1,837, 77.7%; n=1,497/1,926 and 79.4%; n=1,522/1,918, respectively) individuals as well as resolved (71.1%; n=38/53, 73.2%; n=30/41 and 71.7%; n=33/46, respectively) and active (86.7%; n=72/83, 78.6%; n=88/112 and 80.3%; n= 49/61, respectively) HCV coinfections (Fig. 27A).

A high proportion of males in the pre-DAA study were also HCV seropositive (72.8%, n=59/81; P<0.0001) and had resolved (73.7%, n=14/19; P<0.0001) and active (72.6%, n=45/62; P<0.0001) infections. However, in comparison with the pre-DAA study, this study found a significantly higher proportion of DSS obtained from females (14.7%, n=222/1,511 vs 21.3% n=1,301/6,097; P<0.0001) and a significantly lower proportion of HCV seronegative samples obtained from males (85.8%, n=1,220/1,422 vs 78.5%, n=4,785/6,097; p<0.0001) (Fig. 27A).

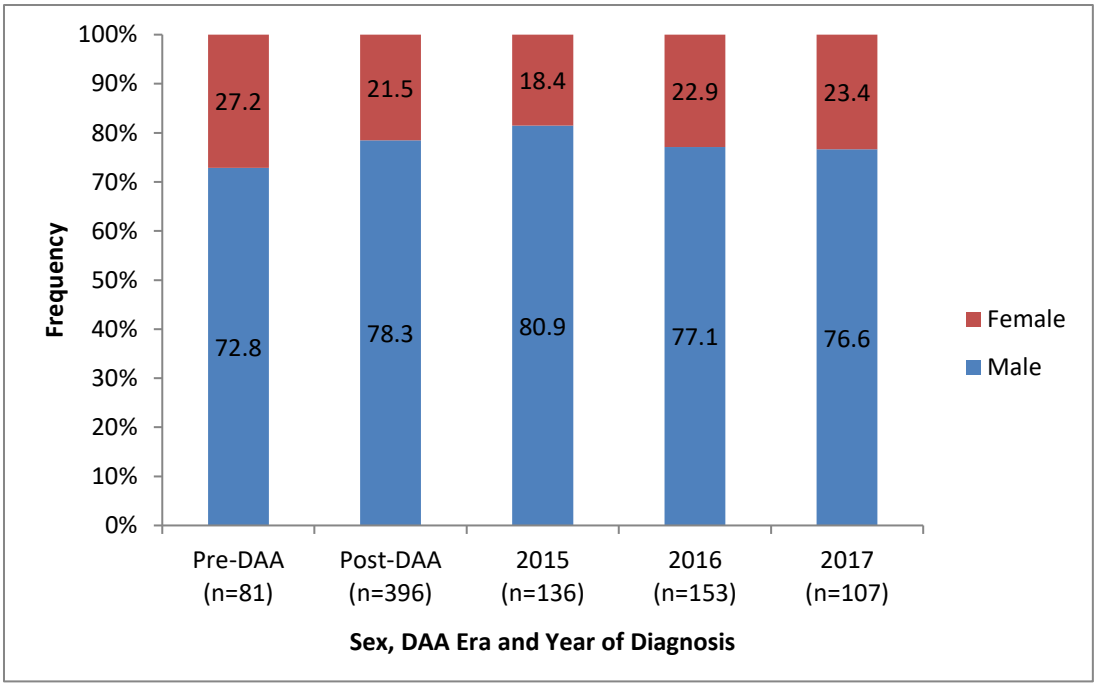
A similar sub-analysis on the contribution of males and females among all HCV coinfections in the different time periods (2015-2017) showed a slight decrease (P>0.05) in males (80.9%, n=110/136; 77.1%, n=118/153 and 76.6%, n=82/107, respectively) and a gradual increase (p>0.05) in females (18.4%, n=25/136; 22.9%, n=35/153 and 23.4%, n=25/107, respectively) (Fig. 27B).

Studies have shown that behavioral and social risks differ between male and female PWIDs (Folch *et al.*, 2013, Tracy *et al.*, 2014) and these differences could attribute to the differences in HCV infection between both sexes (Evans *et al.*, 2003, Tracy *et al.*, 2014). This study, therefore, assessed the contribution of male and female PWIDs among all HCV coinfections in the different time periods. The study found a significantly higher contribution of male PWIDs than female PWIDs in the post (33.8%, n=134/396 vs 8.6%, n=34/396; p<0.0001) and pre-DAA (33.3%, n=27/81 vs 14.8%, 12/81; p<0.01) studies. A further analysis across the diagnoses years 2015-2017 has shown a similar proportion in males (33.8%, n=46/136; 35.3%; n=54/153 and 31.8%; n=34/107, respectively) and a gradual increase in females (5.1%, n=7/136; 9.2%, n=14/153 and 12.1%, n=13/107, respectively) (Fig. 27C). Thus, the analysis shows that male PWIDs contribute around 1/3 of all HCV coinfections in the analyzed time periods, while female PWIDs contribute only roughly 1/10. However, the contribution of female PWIDs to the HCV coinfections is increasing (p=0.44) in the post DAA era.

A.


Male
Female
Not reported
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Frequency
Total DSS (n=1511)
ELISA Reactive (n=81)
ELISA Non-reactive (n=1422)
Resolved Infection (n=19)
Active Infection(n=62)
Total DSS (n=6097)
ELISA Reactive (n=396)
ELISA Non-reactive (n=5681)
Resolved Infection (n=140)
Active Infection (n=256)
Total DSS (n=1980)
ELISA Reactive (n=136)
ELISA Non-reactive (n=1837)
Resolved Infection (n=53)
Active Infection (n=83)
Total DSS (n=2087)
ELISA Reactive (n=153)
ELISA Non-reactive (n=1926)
Resolved Infection (n=41)
Active Infection (n=112)
Total DSS (n=2030)
ELISA Reactive (n=107)
ELISA Non-reactive (n=1918)
Resolved Infection (n=46)
Active Infection (n=61)
Pre-DAA
Post-DAA
2015
2016
2017
DAA Era, Year of Diagnosis and Status of HCV Infection

B.


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Frequency
27.2
21.5
18.4
22.9
23.4
72.8
78.3
80.9
77.1
76.6
Female
Male
Pre-DAA (n=81)
Post-DAA (n=396)
2015 (n=136)
2016 (n=153)
2017 (n=107)
Sex, DAA Era and Year of Diagnosis

C.

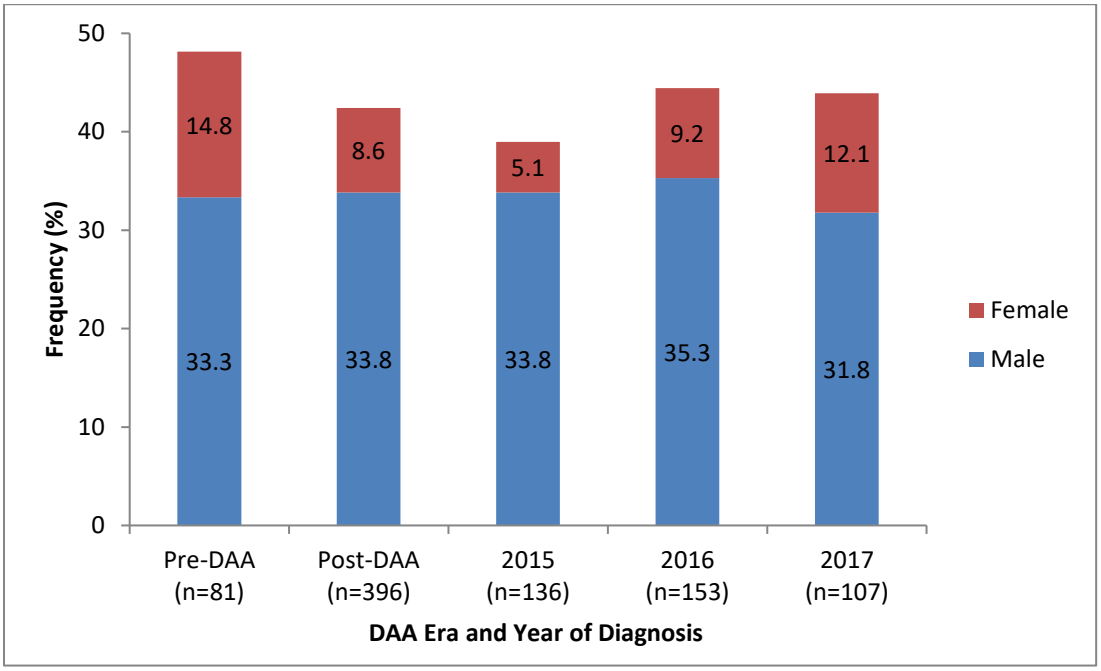
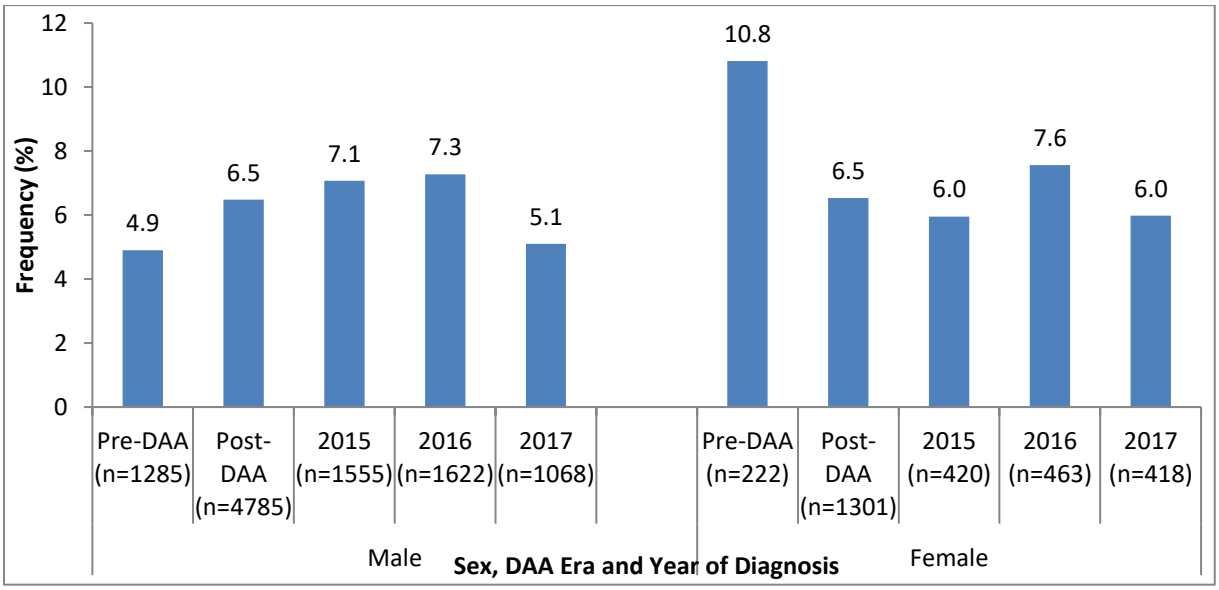


Fig. 27: Contribution of HCV coinfections by sex groups among HIV new diagnoses based on: A) different status of HCV infection B) ELISA reactive samples C) PWIDs of the pre-DAA (2009-2011) and post-DAA (2015-2017) studies.

Analysis of the proportion of HCV coinfection among the tested samples in each of the sex groups indicated an equal proportion of coinfection in both sexes (6.5% each) (Fig. 28A). However, when looking at the coinfected PWIDs that constitute each group, male PWIDs have a slightly higher proportion (78.4%, n=134/171) of HCV coinfections than the female counterparts (75.6%, n=34/45) (Fig. 28B). A further analysis in each of the diagnosis years indicated a similar proportion of coinfection in males in the years 2015 and 2016 (7.1%, n=110/1,555 and 7.3%, n=118/1,622, respectively) and a significant decrease in the year 2017 (5.1%, n=82/1,608, $p<0.05$) (Fig. 28A). Male PWIDs also demonstrate a similar pattern of coinfection (2015: 79.3%, 2016: 81.8% and 2017: 72.3%) (Fig. 28B). On the other hand, the proportion of coinfection in females remained similar in all diagnoses years (6%, each) except for the year 2017 (7.6%, n=35/463) in which case a non-significant increase ($p=0.34$) was observed (comparison year: 2015) (Fig. 28A). In contrast, an increasing proportion ($p=0.1$) of coinfection was observed in the female PWIDs throughout the diagnoses years 2015-2017 (2015: 58.3%, 2016: 77.8% and 2017: 86.7%) (Fig. 28B). A comparison of the current study with the pre-DAA study showed a significant increase in the proportion of coinfection in males (4.9%, n=63/1,285 vs 6.5%, n=310/4,785; $P<0.05$) and a significant decrease in

females (10.8%, n=24/222 vs 6.5%, n=85/1,301; P<0.05) (Fig. 28A). Although not significant, a similar trend was also observed in male PWIDs (73%, n=27/37 vs 78.4%, n=134/171; p=0.47) and female PWIDs (85.7%, n=12/14 vs 75.6%, n=34/45; p=0.42) (Fig. 28B). Overall, male and female PWIDs contribute roughly 3/4 of HCV coinfections in each of the sex groups analyzed in the current and pre-DAA studies.

A.



B.

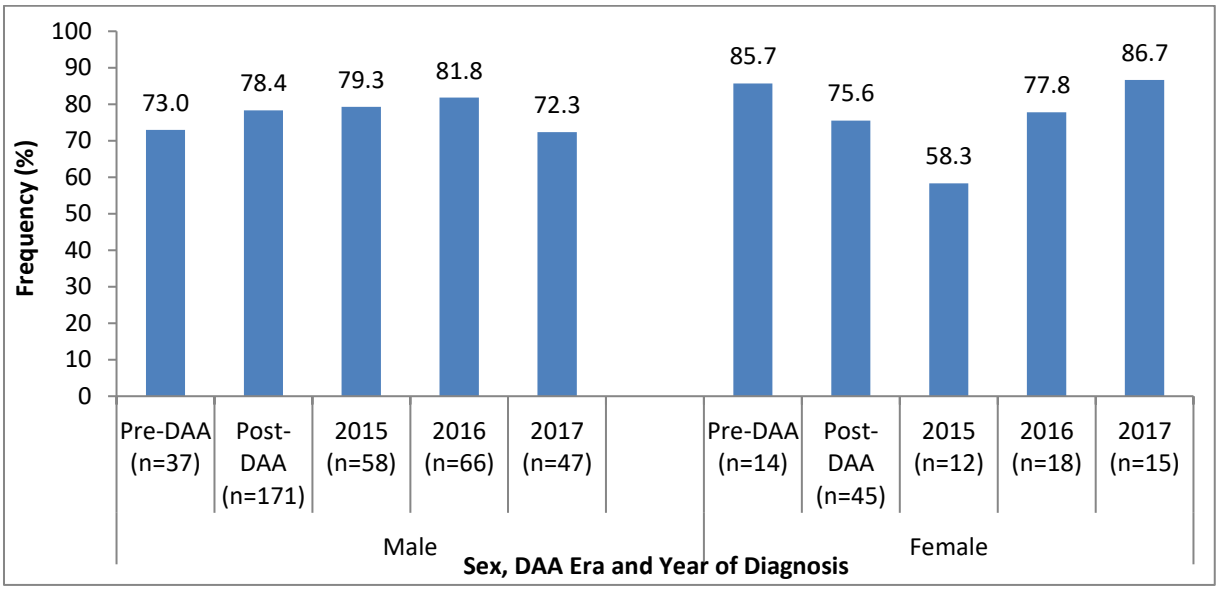


Fig. 28: Proportion of HCV coinfections among HIV new diagnoses in each of the sex groups based on A) ELISA reactive samples, and B) PWIDs of the pre-DAA (2009-2011) and post-DAA (2015-2017) studies.

This study also assessed if there is a difference in the proportion of resolved infections in males and females in the pre-DAA and post-DAA studies. The results show a higher proportion of resolved infections in the post-DAA than pre-DAA study in both males (32.6%, n=101/310 vs 23.7%, n=14/59 vs; p=0.17) and females (45.9%, n=39/85 vs 22.7%, n=5/22; p<0.05), respectively. A further comparison of resolved infections in males and females across the diagnoses years has shown a decrease in the years 2015-2016 (34.5% vs 25.4%; p=0.13 and 60% vs 31.4%; p<0.05, respectively) followed by an increase in the year 2017 (40.2%; p<0.05 and 52%; p=0.11, respectively) (Fig. 29).

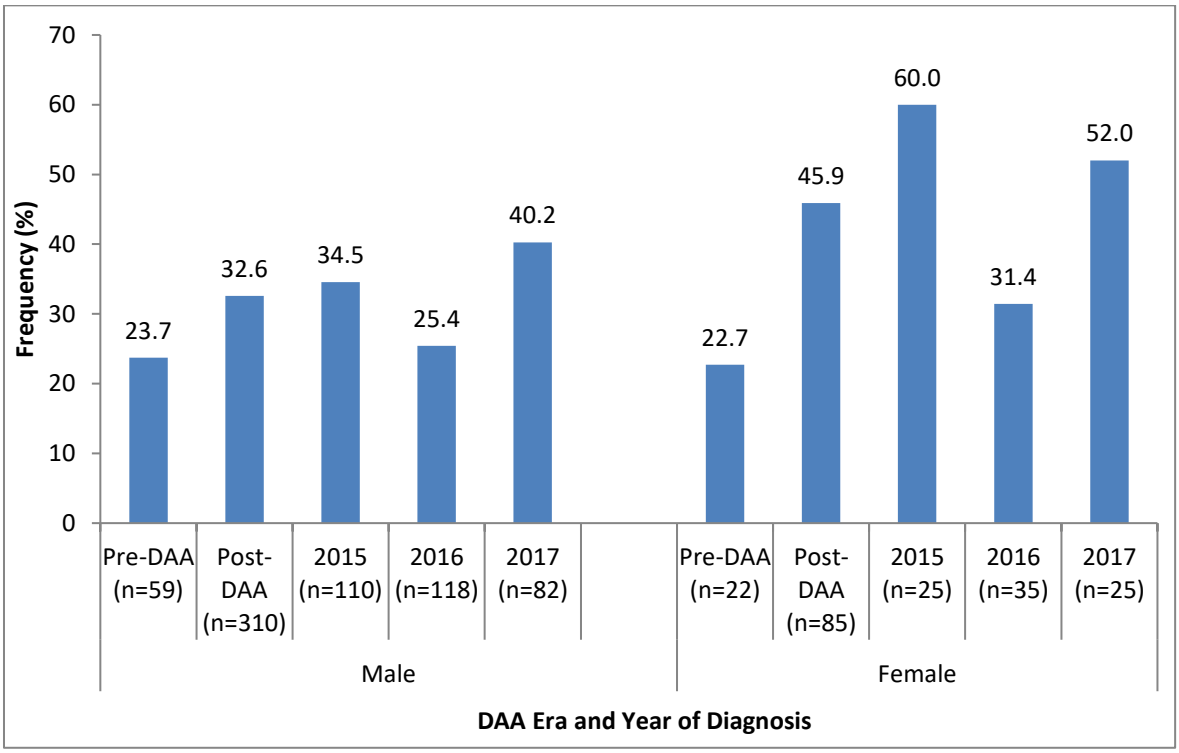


Fig. 29: Proportion of resolved HCV coinfections by sex groups in HIV new diagnoses in the pre-DAA (2009-2011) and post-DAA (2015-2017) studies.

4.3.5 HCV infection by age groups

The proportion of HCV coinfection has been investigated in different age groups of HIV new diagnoses in the current and pre-DAA study. Among the overall studied samples, a similar proportion of age groups was observed in the pre-DAA and post-DAA studies with the age group 30-39 years (30.5%; n=461/1,511 and 32.4%; n=1,978/6,097, respectively) to be the most frequently tested, followed by the age groups 40-49 years (27.4%; n=414/1,511 and 21.1%; n=1,288/6,097, respectively) and 25-29 years (15.3%; n=231/1,511 and 17.3%; n=1,057/6,097, respectively) (Fig. 30).

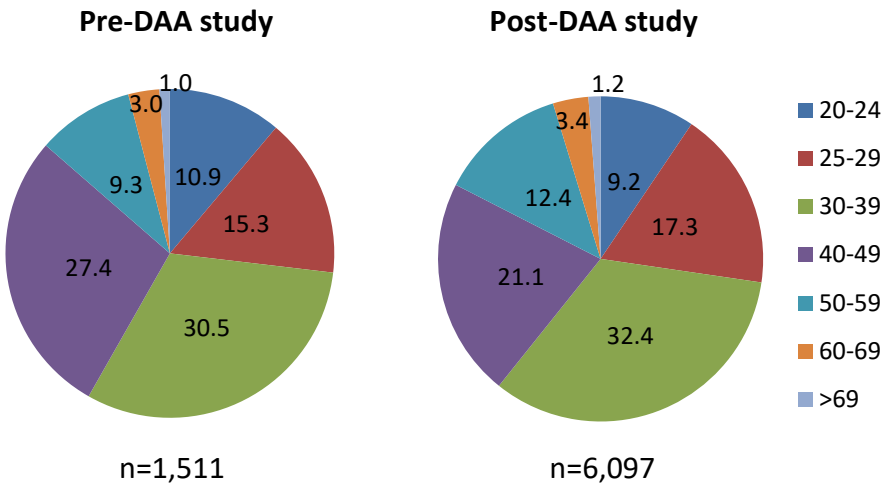


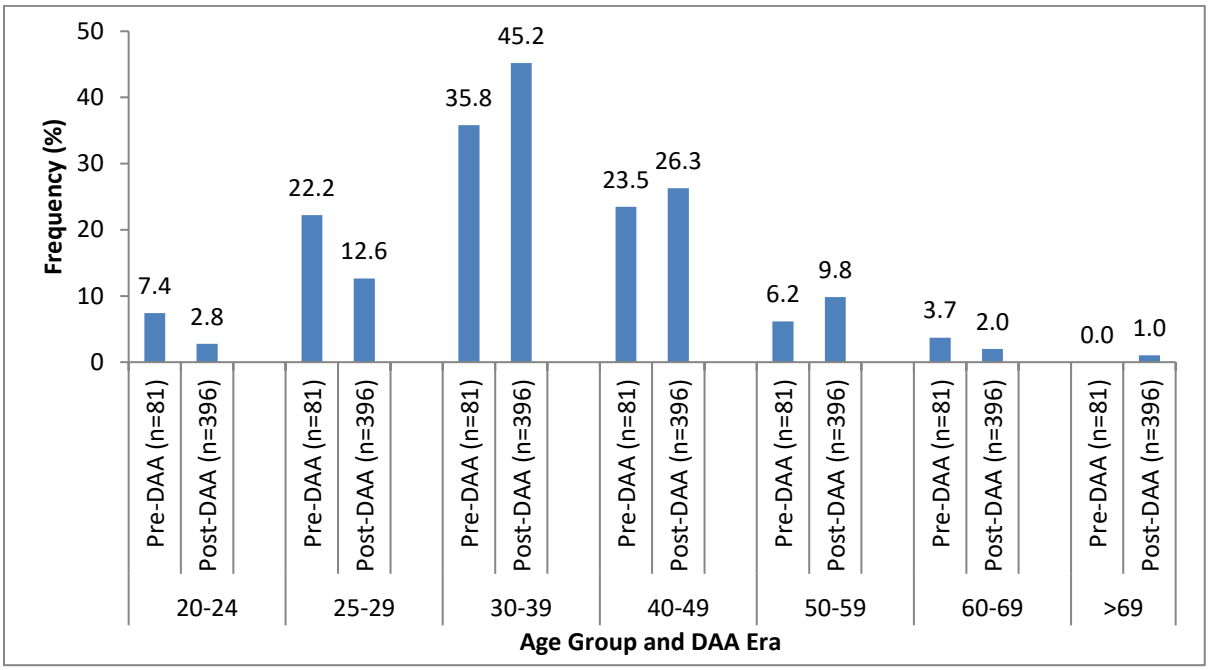
Fig. 30: Proportion of dried serum spots tested in different age groups of pre-DAA (2009-2011) and post-DAA (2015-2017) studies.

In both pre-DAA and post-DAA studies, the largest contribution to the HCV seropositive individuals was made by the age group of 30-39 years old (35.8%, n=29/81 and 45.2%, n=179/396, respectively), followed by the age groups 40-49 years old (23.5%, n=19/81 and 26.3%, n=104/396, respectively) and 25-29 years old (22.2%, n=18/81 and 12.6%, n=50/396, respectively) (Fig. 31A). Moreover, age groups 30-39 years old and 40-49 years old were more likely to have HCV infection than the other age groups (OR=1.76, P<0.0001; and OR=1.32, P<0.05, respectively) (Table 28). Compared with the pre-DAA study, the proportion of HCV infection in the post-DAA study has shown a significant decrease for the age groups 20-24 (p<0.05) and 25-29 (p<0.05) years old. On the other hand, there was an increase in the age groups 30-39 (p=0.12) and 40-49 years old (p=0.6), 50-59 (p=0.31) and >69 (p=0.37) years old (Fig. 31A).

In order to assess whether HCV coinfection in different age groups is consistent with the demographic profile of injecting drug use, this study has compared the contribution of HCV coinfected PWIDs of various age groups to the total HCV infection in pre-and post-DAA studies. Thus, similar to the results shown in Fig. 31A, a higher proportion of coinfection in PWIDs was observed in the age group 30-39 years old (20.5%, n=81/396) followed by the age groups 40-49 (12.1%, n=48/396) and 25-29 years old (6.3%, n=25/396). In comparison with the pre-DAA group, there was a decline in the proportion of HCV infection in all age groups except for the age groups 40-49 (6.2%, n=5/81 vs 12.1%, n=48/396; p=0.12) and 60-69 years old (0%, n=0/81 vs 0.8%, n=3/396; p=0.42). Overall, the analysis shows that PWIDs

of 30-39 years old contribute around 1/5 of all HCV infections in both pre-DAA and post-DAA studies (Fig. 31B).

A.



B.

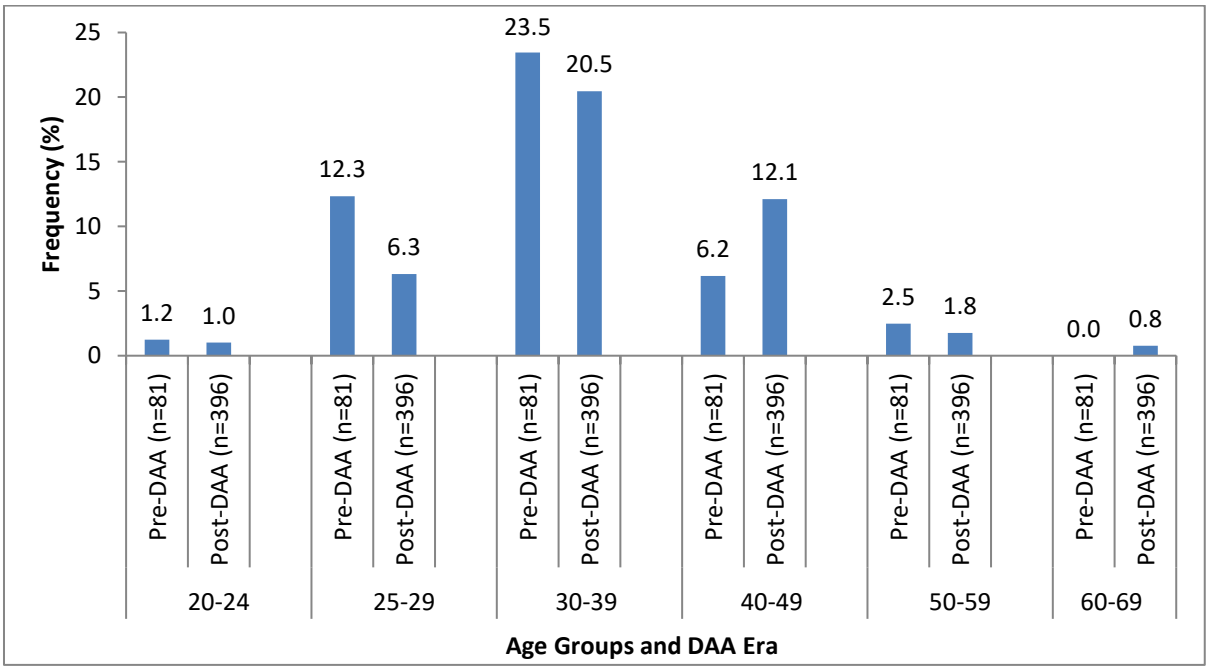


Fig. 31: Contribution of various age groups to all HCV coinfections in HIV new diagnoses based on: A) ELISA reactive samples, and B) PWIDs of the pre-DAA (2009-2011) and post-DAA (2015-2017) studies.

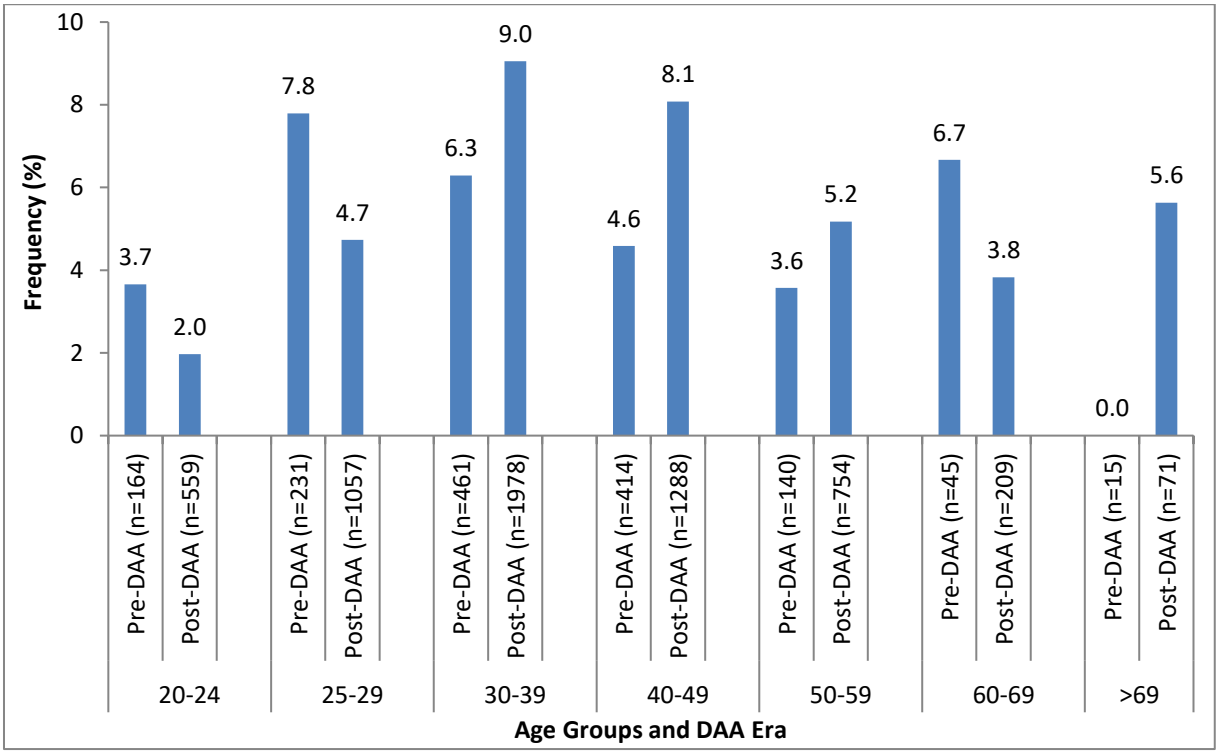
Table 28: Odds ratio for HCV infection by age groups of HIV new diagnosis years 2015-2017.

Age groups (Years)	OR	95% CI	p-value
20-24	0.19	0.09-0.39	p<0.0001
25-29	0.65	0.48-0.89	p=0.0078
30-39	1.76	1.42-2.17	p<0.0001
40-49	1.32	1.04-1.68	p=0.0196
50-59	0.71	0.5-1.01	p=0.0586
60-69	0.57	0.28-1.17	p=0.1295
>69	0.67	0.2-2.09	p=0.4747

Analysis of the proportion of HCV coinfection among the tested samples in each age group shows the largest proportion in the age groups of 30-39 (9%, n=179/1,978) and 40-49 (8.1%, n=104/1,288) years old. Moreover, in comparison with the pre-DAA study, the proportion of HCV infection in the post-DAA study has shown a decrease for the age groups 20-24 (p=0.21), 25-29 (p=0.06) and >69 (P=0.35) years old. On the other hand, there was an increase in the age groups 30-39 (p=0.06) and 40-49 years old (p<0.05), 50-59 (p=0.42) and >69 (p=0.35) years old (Fig. 32A).

This study has also assessed the proportion of HCV coinfected PWIDs in each of the HCV coinfected age groups in the pre- and post-DAA studies. Although, the total number of pre-DAA PWIDs in the age groups of 20-24 (n=1), 50-59 (n=2) and 60-69 (n=0) years old were too low for comparison with the post-DAA PWIDs, the proportion of HCV coinfection in the PWIDs of other age groups was generally high in both studies (50% to 83.3%). In addition, there was a non-significant increase in the proportion of HCV coinfection in the post-DAA PWIDs in the age groups 30-39 (76%, n=19/25 vs 81%, n=81/100; p=0.57) and 40-49 (50%, n=5/10 vs 78.9%, n=45/57; p=0.06) years old (Fig. 32B).

A.



B.

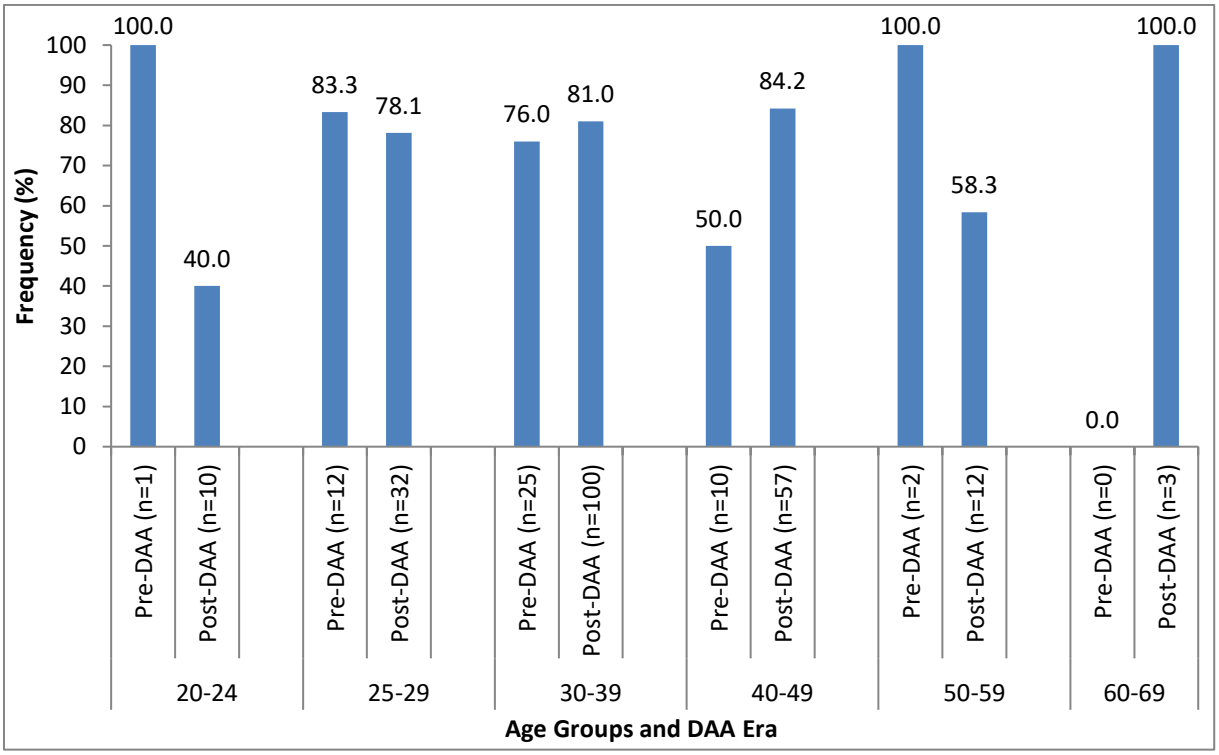
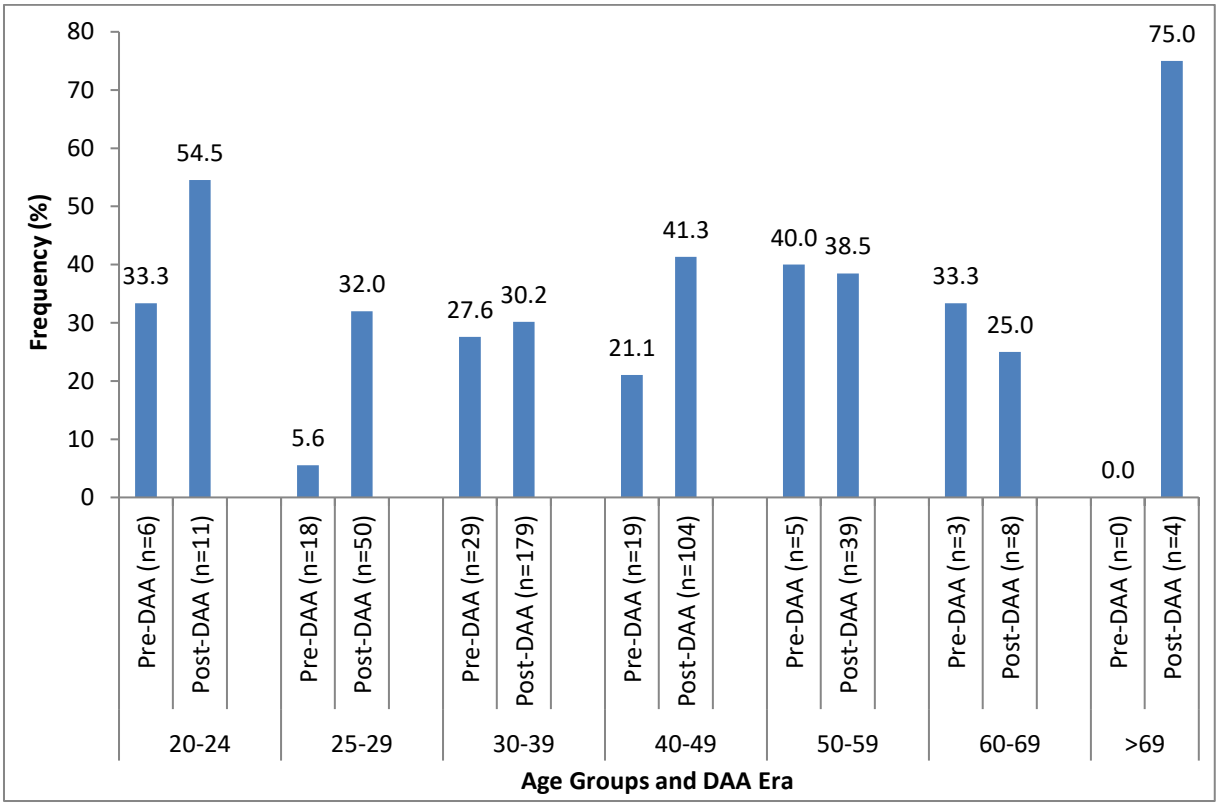


Fig. 32: Proportion HCV infection among HIV new diagnosis in different age groups of A) ELISA reactive samples, and B) PWIDs of the pre-DAA (2009-2011) and post-DAA (2015-2016) studies.

A comparison of HCV resolved infections in the different age groups of pre-DAA and post-DAA studies has shown an increase in the age groups 20-24 (33.3%, n=2/6 vs 54.5%, n=6/11; p=0.41), 25-29 (5.6%, n=1/18 vs 32%, n=16/50; p<0.05) 30-39 (5.6%, n=1/18 vs 30.2%, n=54/179; p=0.77) and 40-49 (21.1%, n=4/19 vs 41.3%, n=43/104; p=0.09) years. Although the total numbers of ELISA reactives in the age groups 50-59 (n=5) and 60-69 (n=3) and >69 (n=0) of the pre-DAA study were too low for comparison with the current study (Fig. 33A), the proportion of resolved HCV infections have been shown to increase (p>0.05) for these age groups and for the age group 40-49 (p>0.05) in the years 2015-2017. Despite a slight decrease in the year 2016 (22.5%; n=16/71), the proportion of resolved infections for the age group 30-39 years old remained similar in the years 2015 and 2017 (35.9%, n=23/64 vs 34.1%, n=15/44, respectively) (Fig. 33B).

A.



B.

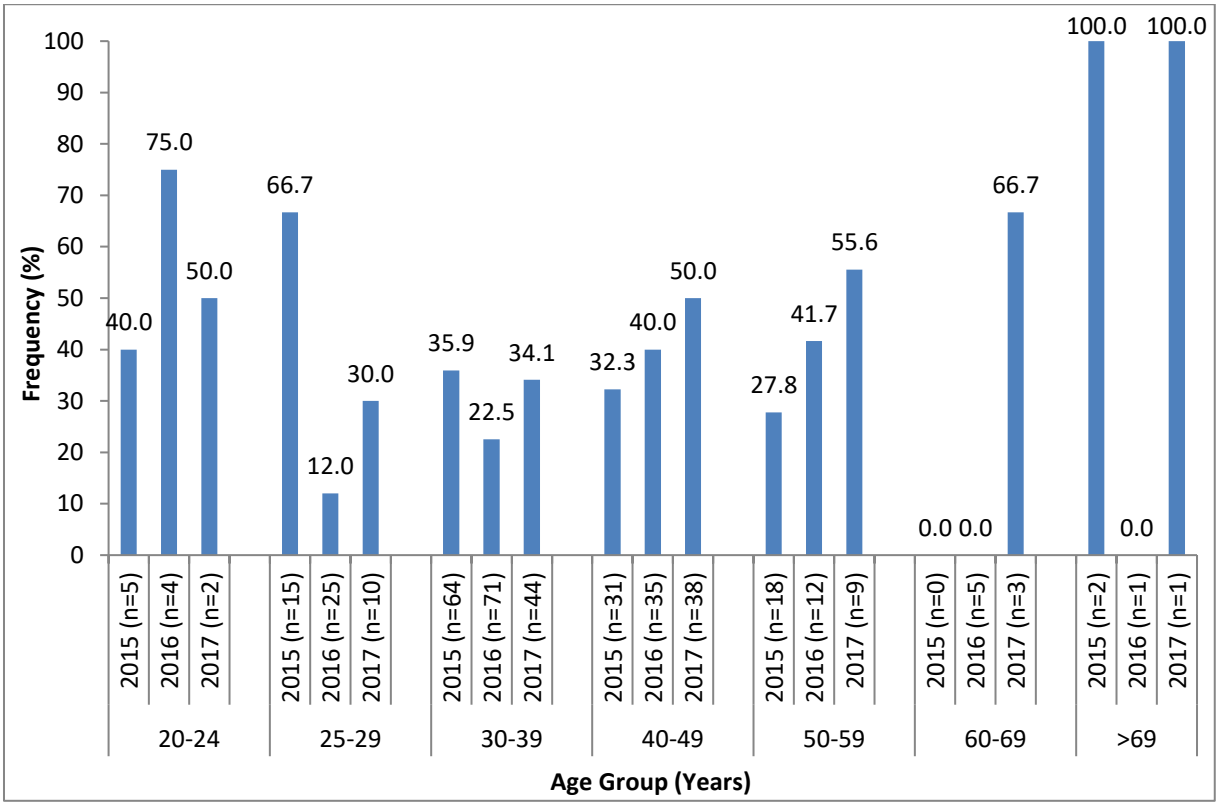


Fig. 33: Proportion of resolved HCV coinfections among HIV new diagnoses in different age groups of A) the pre-DAA and post-DAA studies, and B) diagnoses years 2015-2017.

4.3.6 HCV infection by country of origin

An assessment of HCV coinfection by the country of origin was possible in different time periods based on the statutory anonymus reports provided by the diagnostic laboratories to the RKI. This analysis is particularly important as there has been a huge influx of asylum seekers from high endemic countries in the years 2015 and 2016 (Grote, 2018) which can have an impact on the viremic pool of the country.

From the total 6,097 tested samples, 55% (n=3,355/6,097) of the persons are Germans and 40.6% (n=2,477/6,097) were foreigners by origin. For the remaining 4.4% (n=265/6,097) of the persons, the country of origin is not reported (Fig. 34). Germans also constitute a higher proportion than foreign nationals in the diagnoses years 2015 (53.2%, n=1,054/1,980 vs 43.6%, n=863/1,980), 2016 (57.5%, n=1,199/2,087 vs 38.7%, n=808/2,087) and 2017 (54.2%, n=1,102/2,030 vs 39.7%, n=806/2,030) (Fig. 35). A similar order in the proportion of Germans (62.3%; n=941/1,511) and foreign nationals (23.2%; n=351/1,511) was also reported in the pre-DAA study (Fig. 34).

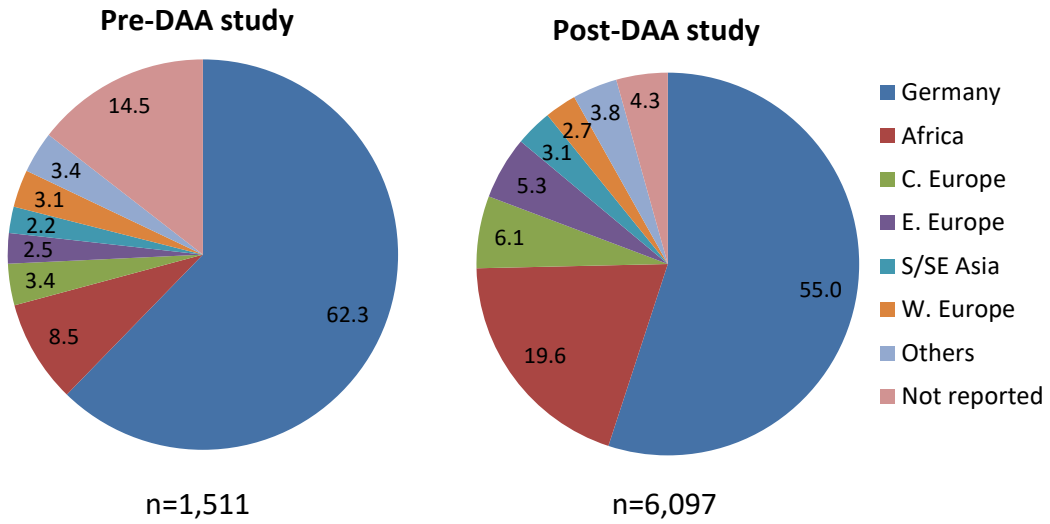


Fig. 34: Proportion of regional origins of different nationals among dried serum spots tested in the pre-DAA (2009-2011) and post-DAA (2015-2017) studies.

In the pre-DAA and post-DAA studies, people from Africa (8.5%; n=129/1,511 and 19.6%; n=1,196/6,097, respectively) and Central Europe (3.4%; n=52/1,511 and 6.1%; n=372/6,097, respectively) represent the largest group of persons with a foreign origin (Fig. 34). In the same manner, people from the two regions contribute the largest share of foreign nationals in the diagnoses years 2015-2017 (Fig. 35).

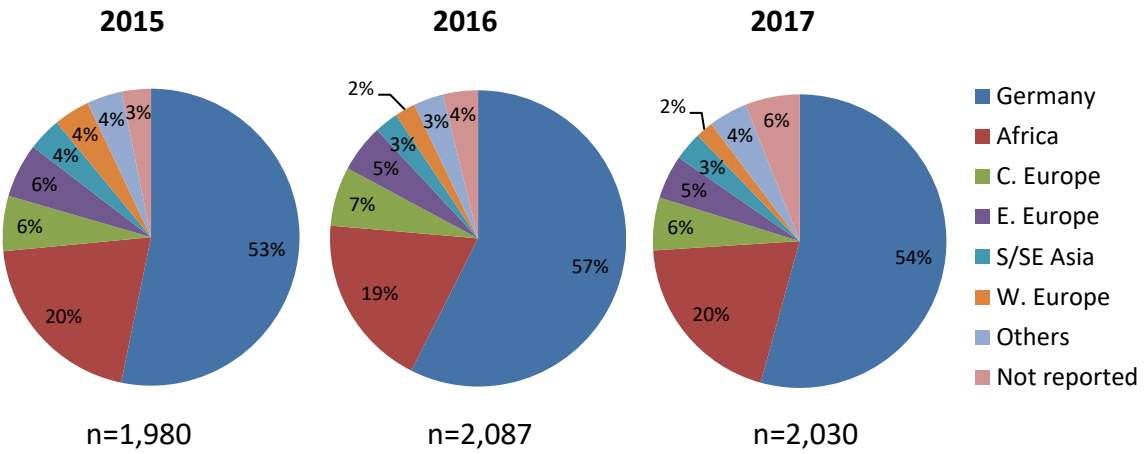


Fig. 35: Proportions of regional origins of different nationals among dried serum spots tested in the diagnoses years 2015-2017.

Compared to Germans, foreign nationals had a significantly higher proportion of HCV seropositive individuals (8.8%, n=219/2,477 vs 4.6%, n=156/3,355; p<0.0001). Moreover, foreign nationals are more likely to have HCV infection than German nationals (OR=4.47,

P<0.0001) (Table 29). In consistent with this study, the pre-DAA study also showed foreign nationals compared to Germans to have a significantly higher proportion of HCV seropositive individuals (8%, n=28/351 vs 4.4%, n=41/941; p<0.05) (Fig. 36).

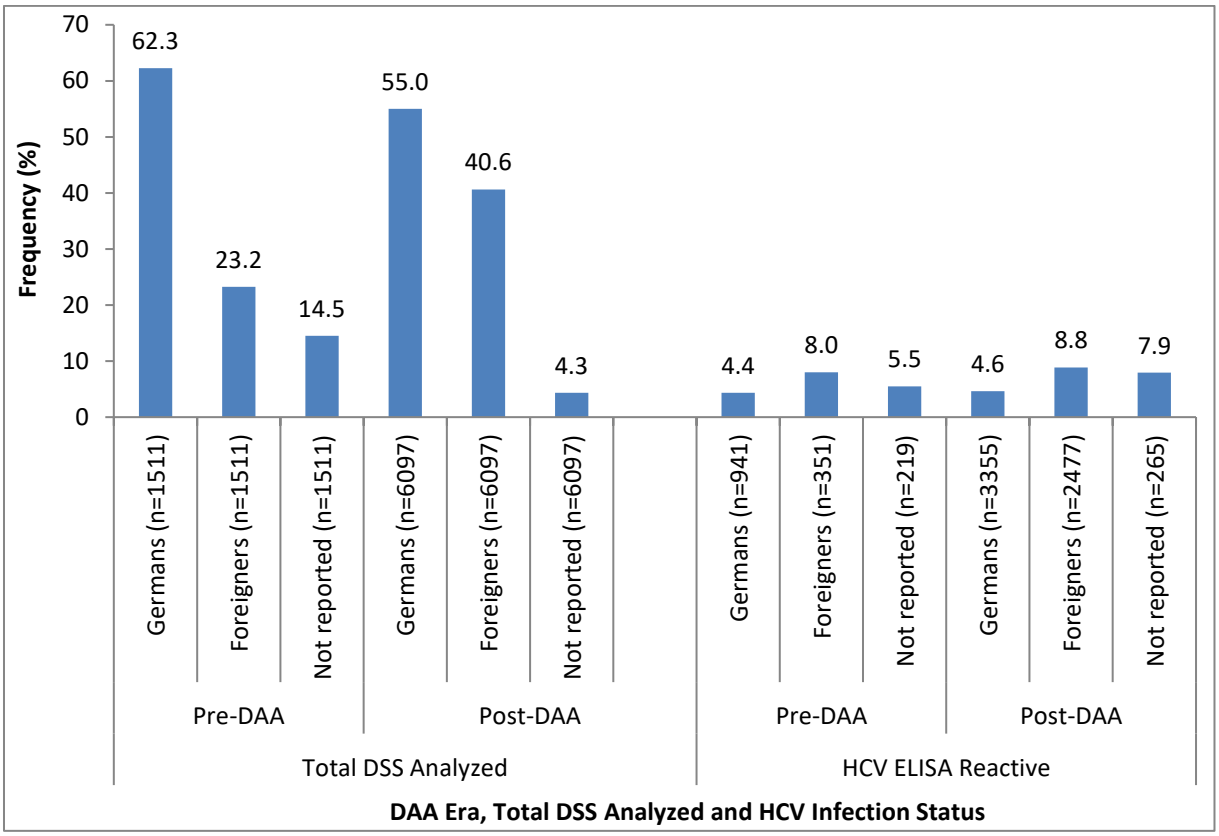


Fig. 36: Origin and status of HCV infection in all DSS samples and among HIV new diagnoses in the pre-DAA (2009-2011) and post-DAA (2015-2017) studies.

Among the foreign nationals, the highest proportion of HCV coinfection was found in the people from E. Europe (38.3%) and C. Europe (11.3%). In comparison to the pre-DAA study, in this study, the proportion of HCV infection has slightly increased in the people from foreign counties (8%; n=28/351 vs 8.8%; n=219/2,477; p=0.61), S/SE Asia (6.1%; n=2/33 vs 8.4%; n=16/190; p=0.65), W. Europe (2.1%; n=1/47 vs 6.1%; n=10/164; p=0.27) and C. Europe (9.6%; n=5/52 vs 11.3%; n=42/372; p=0.71). For German nationals, the proportion of infection was nearly similar in both studies (4.4%; n=41/941 vs 4.6%; n=156/3,355; p=0.79). A decrease in the proportion of infection was also noted in people from Africa (2.3%; n=3/129 vs 1.9%; n=23/1,196; p=0.75) and E. Europe (42.1%; n=16/38 vs 38.3%; n=124/324; p=0.64) (Fig. 37). In addition, foreign nationals and people from East Europe are more likely

to have HCV infection than other nationalities (OR=4.47, P<0.0001 and OR=4.18, P<0.0001, respectively) (Table 29).

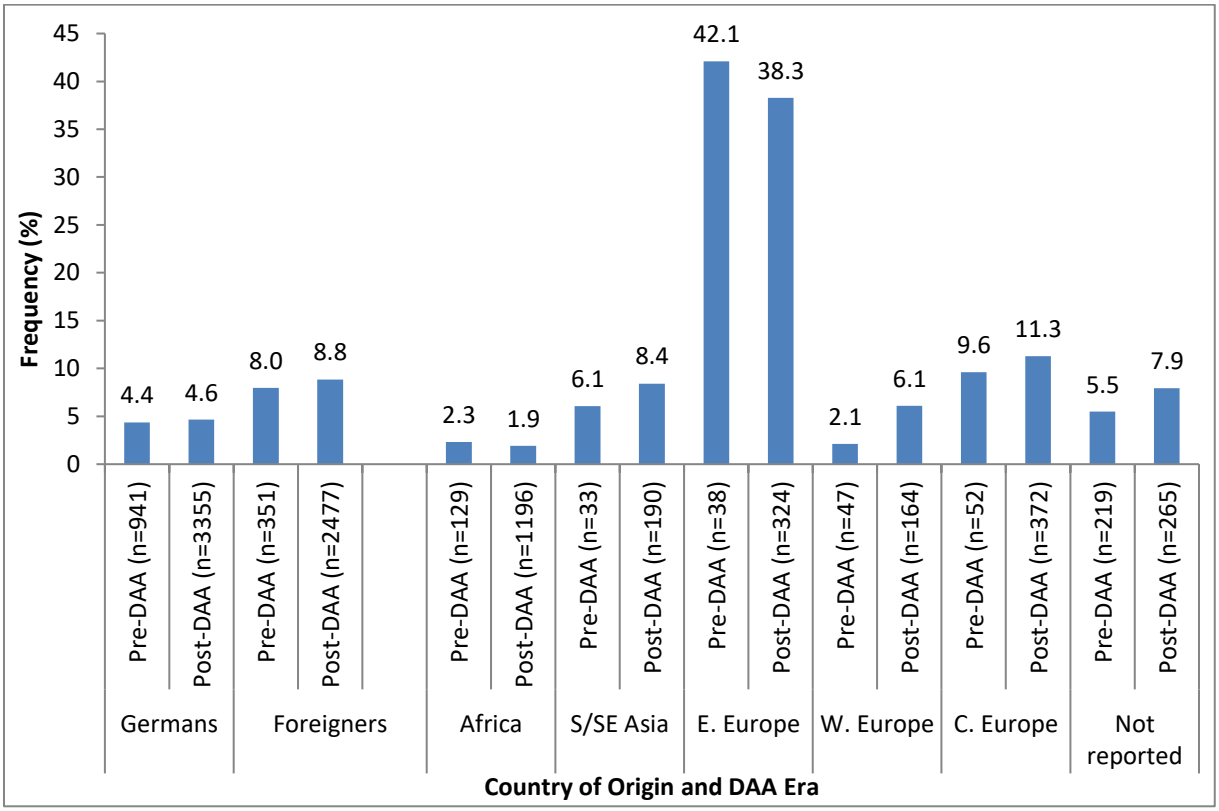


Fig. 37: Proportion of HCV infections by country of origin among HIV new diagnoses in the pre-DAA (2009-2011) and post-DAA (2015-2016) studies.

Table 29: Odds ratio for HCV infection in different nationalities of HIV new diagnosis years 2015-2017.

Country of origin	OR	95% CI	p-value
German	0.22	0.17-0.28	p<0.0001
Foreigner	4.47	3.51-5.71	p< 0.0001
Africa	0.04	0.02-0.06	p< 0.0001
S/SE Asia	0.47	0.28-0.8	p=0.0053
E. Europe	4.18	3.22-5.44	p<0.0001
W. Europe	0.33	0.17-0.63	p=0.0009
C. Europe	0.64	0.46-0.91	p=0.0129

As depicted in Fig. 38, the proportion of HCV infection has decreased throughout the diagnoses years 2015 to 2017 in foreign nationals (11.4%; n=90/787, 10.1%; n=75/740 and 7.7%; n=54/699, respectively) and in people from S/SE Asia (13.9%; n=10/72, 5.6%; n=3/54 and 4.7%; n=3/64, respectively), E. Europe (41.9%; n=49/117, 37.6%; n=41/109 and 34.7%; n=34/98, respectively) and C. Europe (13.4%; n=16/119, 13.3%; n=18/135 and 8.2%; n=8/98, respectively). In Germans, compared to the diagnosis year 2015, the proportion has shown a significant increase in the year 2016 (3.8%, n=40/1,054 vs 6%, n=72/1,199; $p<0.05$) followed by a significant decrease in the year 2017 (4%, n=44/1,102; $p<0.05$). On the other hand, in comparison to the diagnosis year 2015, the proportion of HCV infection has shown a significant decrease in foreign nationals during the diagnosis year 2017 (11.4% vs 7.7%; $p<0.05$).

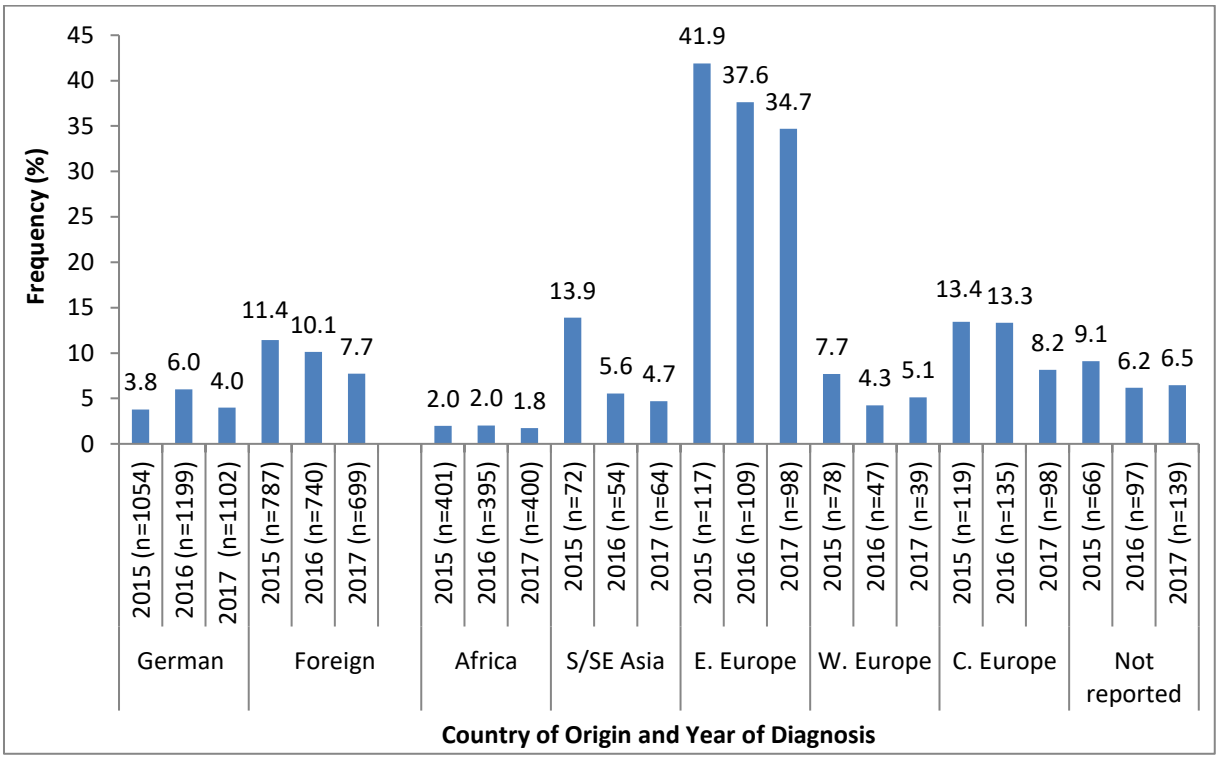


Fig. 38: Proportion HCV infection by country of origin among HIV new diagnoses years 2015-2017.

Analysis of transmission groups among different nationalities has shown PWIDs to be the dominant group in all nationalities, except for the people from Africa and West Europe in which case the dominant groups were heterosexuals (69.6%, n=16/23; $p<0.01$) and MSM (50%, n=5/10; $p<0.05$), respectively. In Germans, MSM (33.3%, n=52/156; $p<0.01$) also

accounts as a second major transmission group next to the PWIDs (44.2%, n=69/156; p=0.51) (Fig. 39).

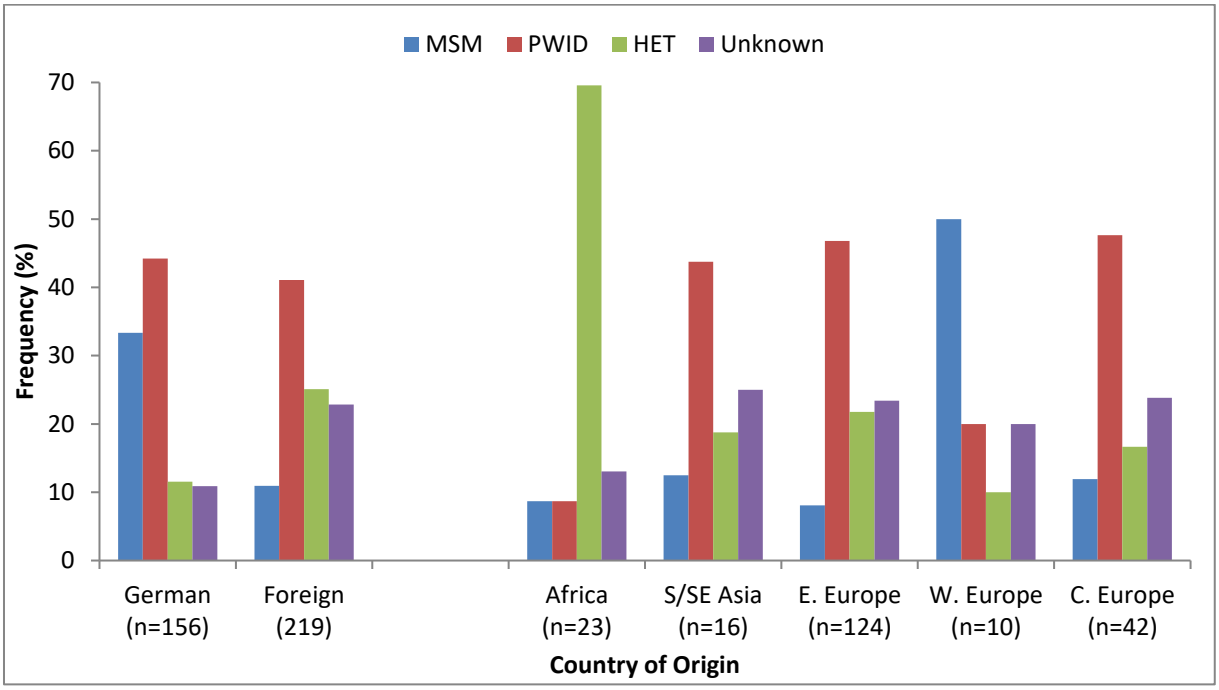


Fig. 39: The proportion of different transmission groups by country of origin in HCV seropositive individuals in the HIV new diagnoses years 2015-2017.

As PWIDs contribute to the largest share of HCV coinfection in German and foreign nationals (Fig. 39), a comparison with PWIDs from the pre-DAA study was made in order to find out the change in the level of coinfection in the post-DAA era. In this study, the proportion of HCV coinfection was generally high in the PWIDs of all nationalities (40%-87.5%). Although there were smaller numbers of samples for some groups of the pre-DAA study, a comparison with this study shows a similar proportion in German nationals (76%, n=19/25 vs 75%, n=69/92; p=0.92) and an increase in foreign (72.2%, n=13/18 vs 78.9%, n=90/114; p=0.53), African (33.3%, n=1/3 vs 40%, n=2/5; p=0.86) and E. European (80%, n=8/10 vs 85.3%, n=58/68; p=0.67) nationals (Fig. 40).

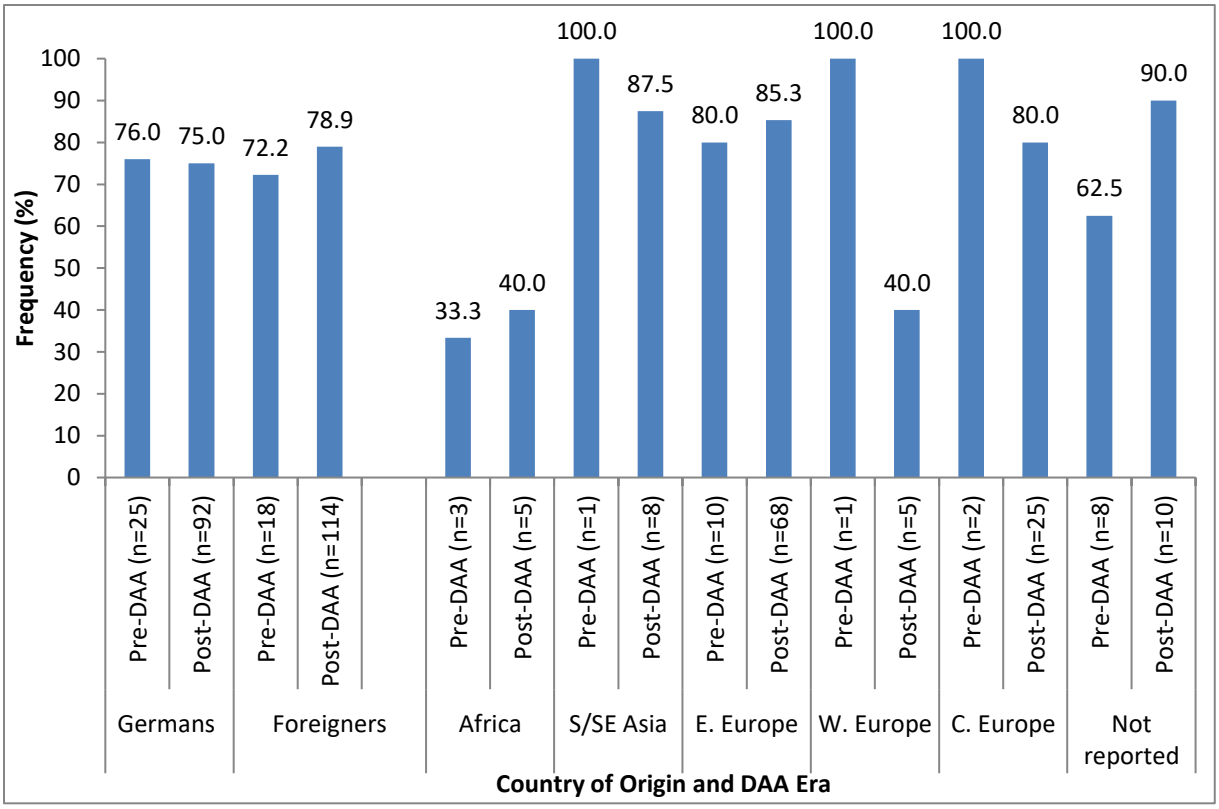
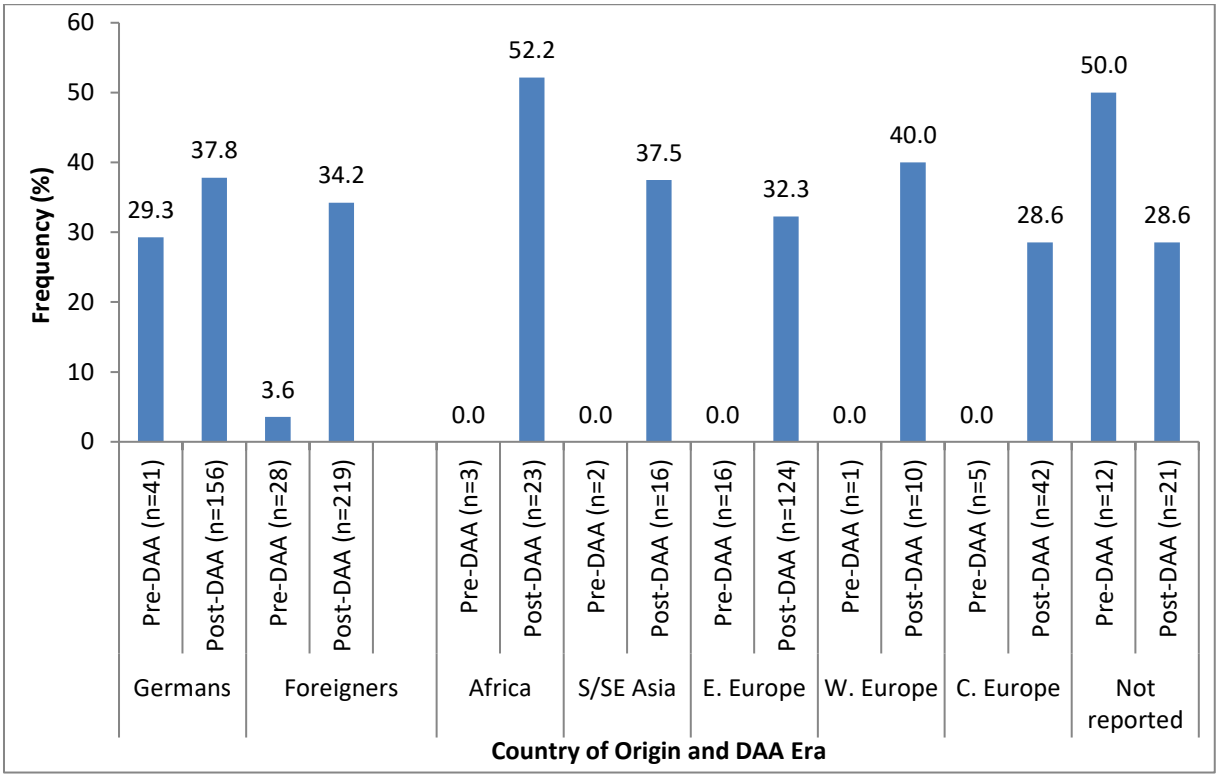


Fig. 40: Proportion of injection drug users by their origin to the HCV coinfected cases among HIV newly diagnoses of pre-DAA (2009-2011) and post-DAA (2015-2017) studies.

A comparison in the proportion of HCV resolved infections was made with the pre-DAA study in order to assess the changes in these cases after the introduction of DAAs. Thus, in comparison with the pre-DAA study, the proportion of HCV resolved infection in the post-DAA study has shown an increase in all nationalities. The increase in the proportion of resolved infection was particularly significant in people of foreign (3.6%, n=1/28 vs 34.2%, n=75/219; p<0.01) and Eastern European (0%, n=0/16 vs 32.3%, n=40/124; p<0.01) origin (Fig. 41A).

A further comparison of resolved infections among the diagnoses years (2015-2017) has shown a decrease in the diagnoses years 2015-2016 in Germans (40%, n=16/40 Vs 36.1%, n=26/72; p=0.68), foreigners (40%, n=36/90 vs 20%, n=15/75; p<0.01) and Eastern Europeans (32.7%, n=16/49 vs 22%, n=9/41; p=0.26) followed by an increase in the year 2017 (38.6%, n=17/44; p=0.78, 44.4%, n=24/54; p<0.01 and 44.1%, n=15/34; p<0.05, respectively). However, a gradual and a non-significant decrease (p>0.05) was observed in Africans throughout the study period (75%; n=6/8, 50%; n=4/8 and 28.6%; n=2/7, respectively) (Fig. 41B).

A.



B.

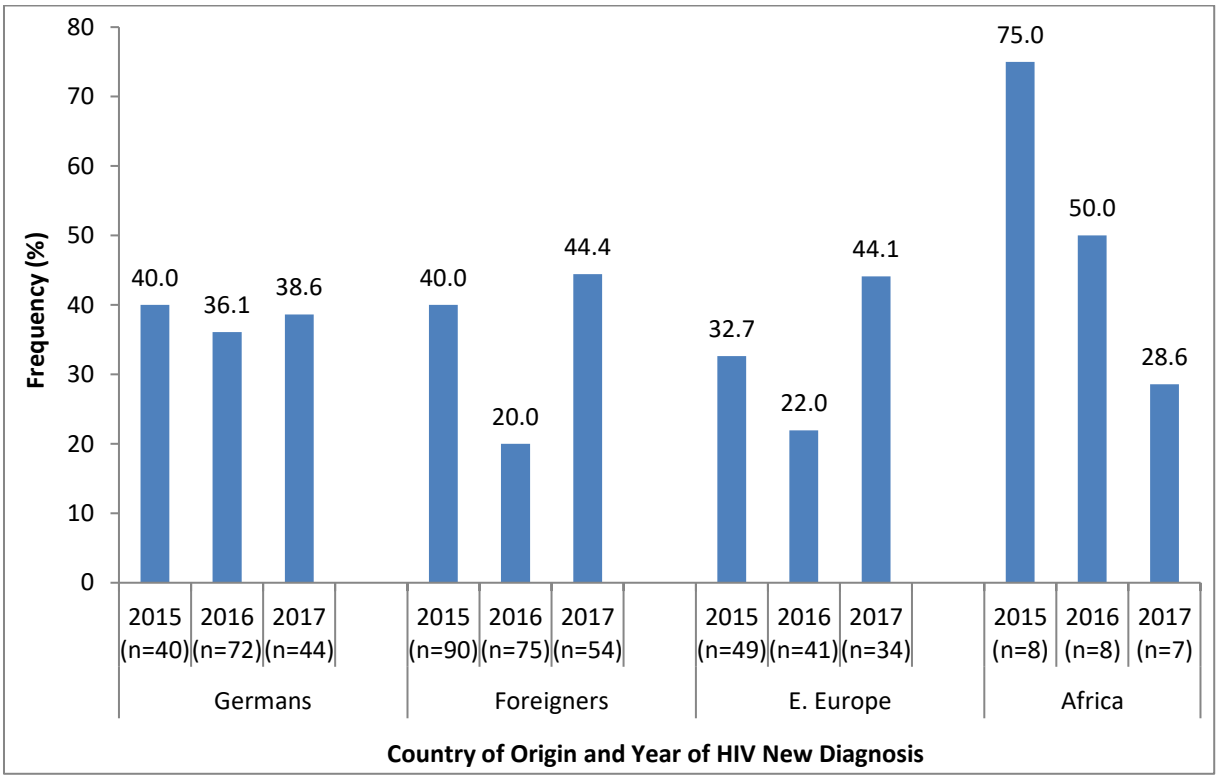


Fig. 41: Proportion of resolved HCV coinfections by country of origin among HIV new diagnoses in A) the pre-DAA and post-DAA studies, and B) diagnoses years 2015-2017.

4.3.7 Genotype and subtype distribution

For the diagnoses years 2015-2017, the genotype and subtype could be successfully determined for 91.1% (n=233/256) of samples with an active HCV infection. For 6.6% (n=17/256) of the samples, the genotype and subtype could not be successfully tested with a nested PCRs in the NS5B region of the HCV genome and for the remaining 2.3% (n=6/256) there was no DSS to carry out the nested PCRs. Overall, 23 of the 256 qPCR-positive samples (9%) could not be geno-/subtyped. The viral load of samples that were successfully geno-/subtyped ranged from 3.45E + 01(IU/ml) to 4.05E + 07 (IU/ml). The mean viral load was 1.59E + 06 (95%CI: 1.1E + 06 IU/ml - 2.08E + 06 IU/ml).

Within the overall studied samples in the post-DAA study (n=233), genotype 1 was the most frequently observed with 57.1% (subtype 1a: 33.9%, subtype 1b: 22.3%, subtype 1e: 0.4% and an unidentified subtype: 0.4%) followed by genotype 3 with 33.5% (exclusively subtype 3a), genotype 4 with 7.7% (subtype 4a: 3%, subtype 4b: 0.4%, subtype 4d: 3.9% and subtype 4k: 0.4%) and genotype 2 with 1.7% (subtype 2a: 1.3% and subtype 2 a/b: 0.4%). For the pre-DAA group, 47/62 active HCV infections were successfully genotyped. Similar to this study, genotype 1 was the most frequently observed with 55% (subtype 1a: 38% and subtype 1b: 17%) followed by genotype 3 with 30% (exclusively subtype 3a). Although statistically not significant, there was a decrease in the proportion of genotype 1 infections in this study compared to the pre-DAA study (70.2% vs 57.1%; p=0.09) (Fig. 42).

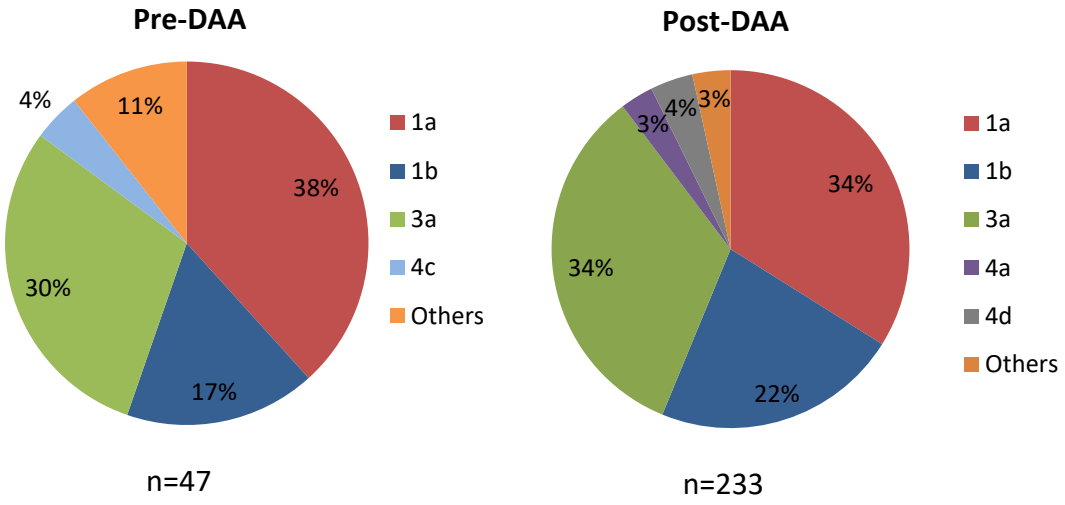


Fig. 42: The overall subtype distribution of active HCV infections in the pre-DAA (2009-2011) and post-DAA (2015-2016) studies.

The proportion of each subtype was relatively similar across the years 2015-2017. However, in comparison to the year 2015, a significant increase in the proportion of genotype 1 infection (47.2%; n=34/72 vs 63.7%; n=65/102; p<0.05) and a decrease in the proportion of genotype 3 (subtype 3a) infection (39%; n=28/72 vs 30%; n=31/102; p=0.21) has been observed in 2016 (Fig. 43).

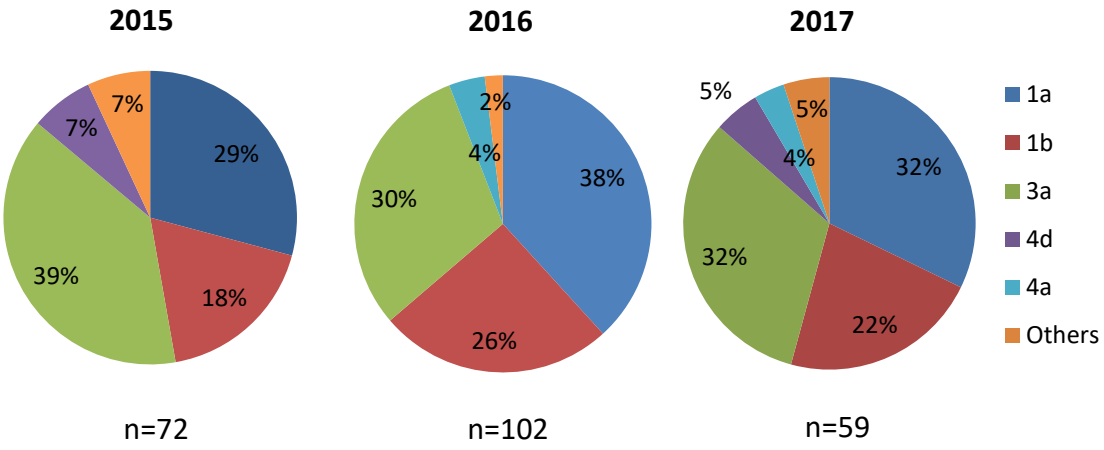


Fig. 43: Subtype distribution in the active HCV infections from the HIV diagnoses years 2015-2017.

Analysis of HCV subtypes in different transmission groups revealed a significant association of subtype 1a with MSM (57.5%; n=23/40; p<0.01). Although not significant, there were relatively high proportions of subtype 3a (39.3%; n=42/107; p=0.12) and subtype 1a (33.6%; n=36/107; p=0.06) in PWIDs as well as subtype 3a (42.9%; n=15/35; p=0.24) and subtype 1b (34.3%; n=12/35; p=0.08) in heterosexuals. In comparison to the pre-DAA study, subtype 4c has shown a significant decrease in MSM (20%, n=2/10 vs 0%, n=0/40; p<0.01). In a similar comparison, although not significant, an increase in the proportions of subtype 1a in MSM (40%, n=4/10 vs 57.5%, n=23/40; p=0.32) and subtype 1b in heterosexuals (20%, n=2/10 vs 12.5%, n=5/40; p=0.54) was also observed in this study (Fig. 44).

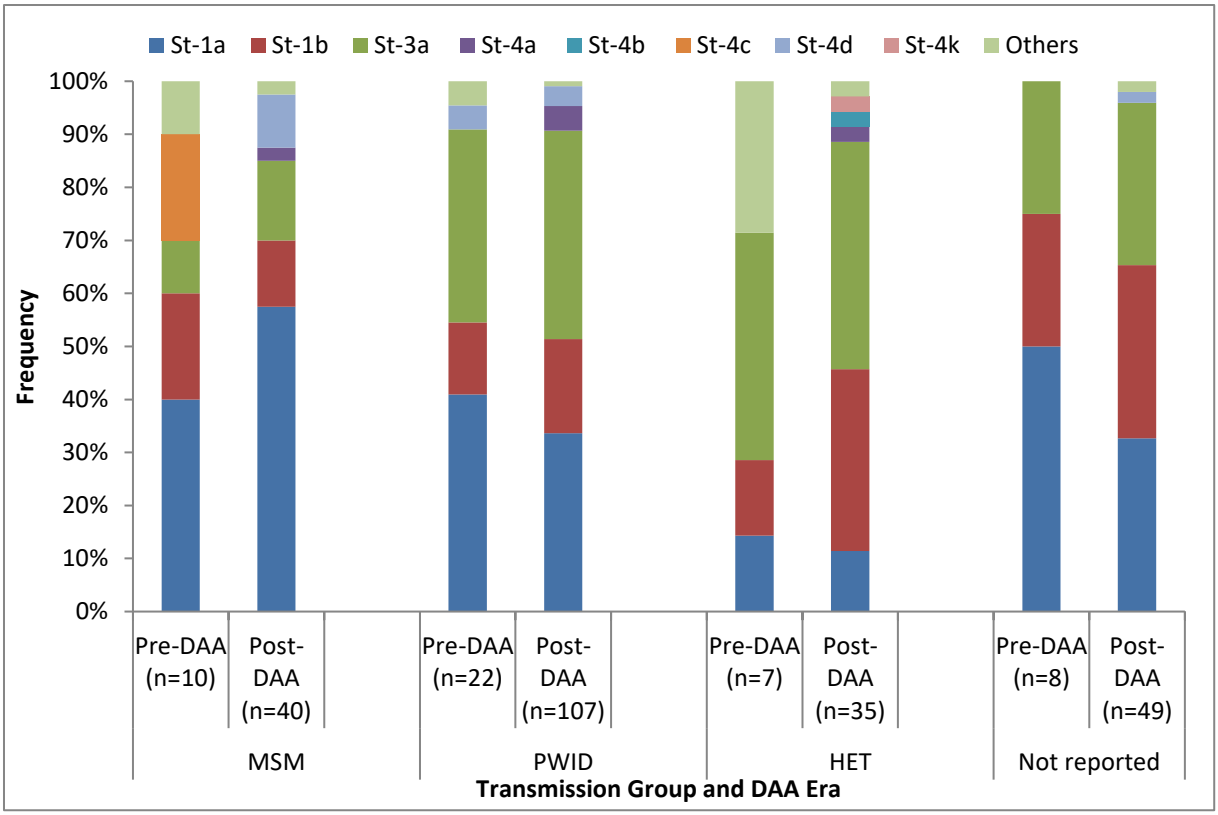


Fig. 44: Proportion of HCV subtypes among HIV new diagnosis in different transmission groups of the pre-DAA (2009-2011) and post-DAA (2015-2016) studies. St = Subtype.

Analysis of HCV subtypes by country of origin in this study shows a significant association of subtype 1a with Germans (50%, n=43/86; p<0.01) as well as subtype 1b (30.5%, n=40/131; p<0.05) and subtype 3a (39.7%, n=52/131; p<0.01) with foreigners. In comparison with the pre-DAA study, a significant decrease of subtype 4c (9.5%, n=2/23 vs 0%, n=0/86; p<0.01) and a non-significant increase of subtype 1b (0%, n=0/23 vs 11.6%, n=10/86; p=0.09) were observed among German nationals. A non-significant increase of subtype 1a (13.6%, n=0/23 vs 22.1%, n=29/131; p=0.35) was also observed among foreign nationals in the post-DAA group (Fig. 45).

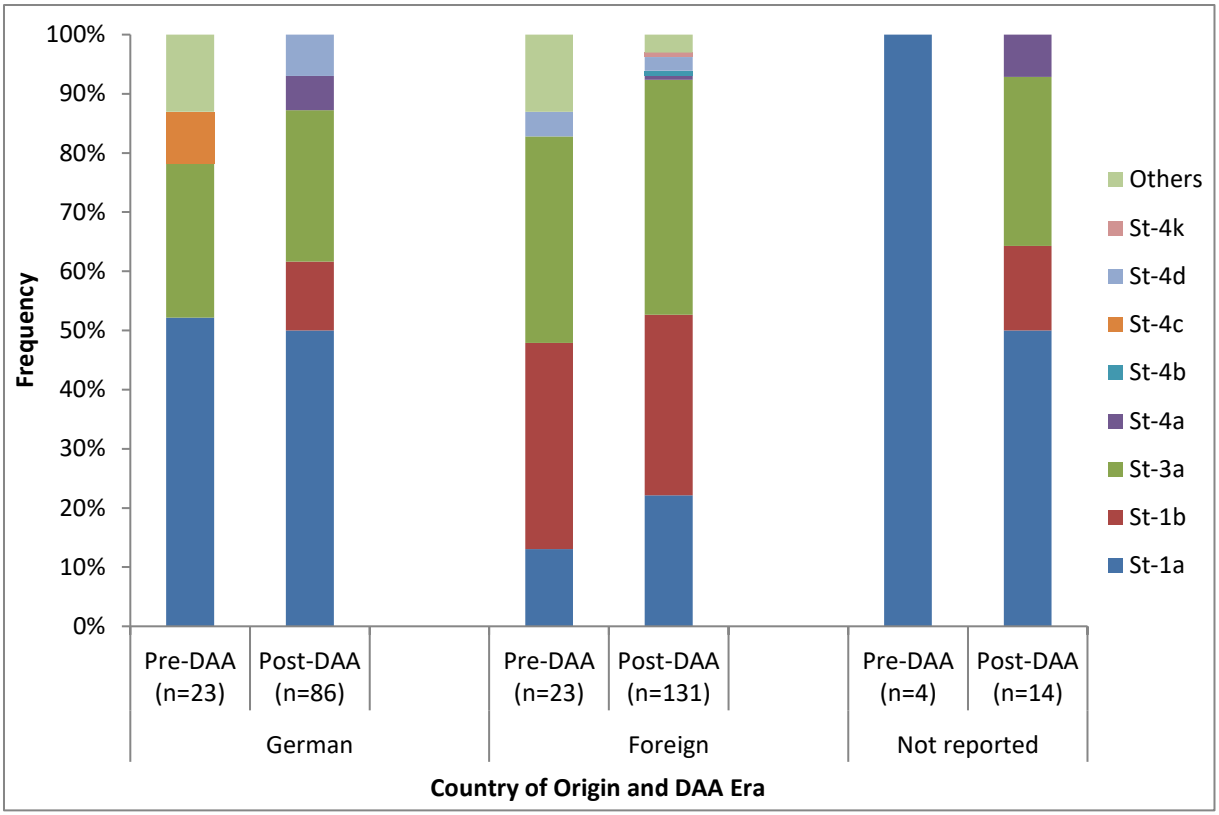


Fig. 45: Proportion of HCV subtypes among HIV new diagnoses in Germans and foreigners in the pre-DAA (2009-2011) and post-DAA (2015-2016) studies.

4.3.8 Analysis of resistance associated substitutions

The evaluation and interpretation of existing RAS was performed using a web tool (geno2pheno). HCV sequences were classified into the corresponding genomic regions as sensitive, reduced susceptibility and resistant to the individual drugs approved for the subtype. In addition, RAS indicating substitution to the wild type reference sequence, but not associated with decreased susceptibility to DAAs (herein after referred to as "DAA-relevant position substitution") were included in the analysis.

The following analysis includes only samples for which sequence information is available on all DAA-relevant genome regions (n=160). In each of the figures below, the highest type of resistance against DAAs is shown. If, for example, two RAS are observed within a single sample, one which leads to reduced susceptibility and one leading to resistance, only the RAS leading to resistance were considered.

Based on all classes of medication, the total RAS proportion was found to decrease from 69% (n=29/42) in the diagnosis year 2015 to 60.7% (n=48/79) and 56.4% (n=22/39) in the

diagnoses years 2016 and 2017, respectively. The proportion of resistance at a DAA-relevant position was similar for the diagnoses years 2015 and 2016 (21.4%; n=9/42 and 21.5%; n=17/79, respectively). However this has shown a decline (p=0.25) to 12.8% (n=5/39) in the diagnosis year 2017 (Fig. 46).

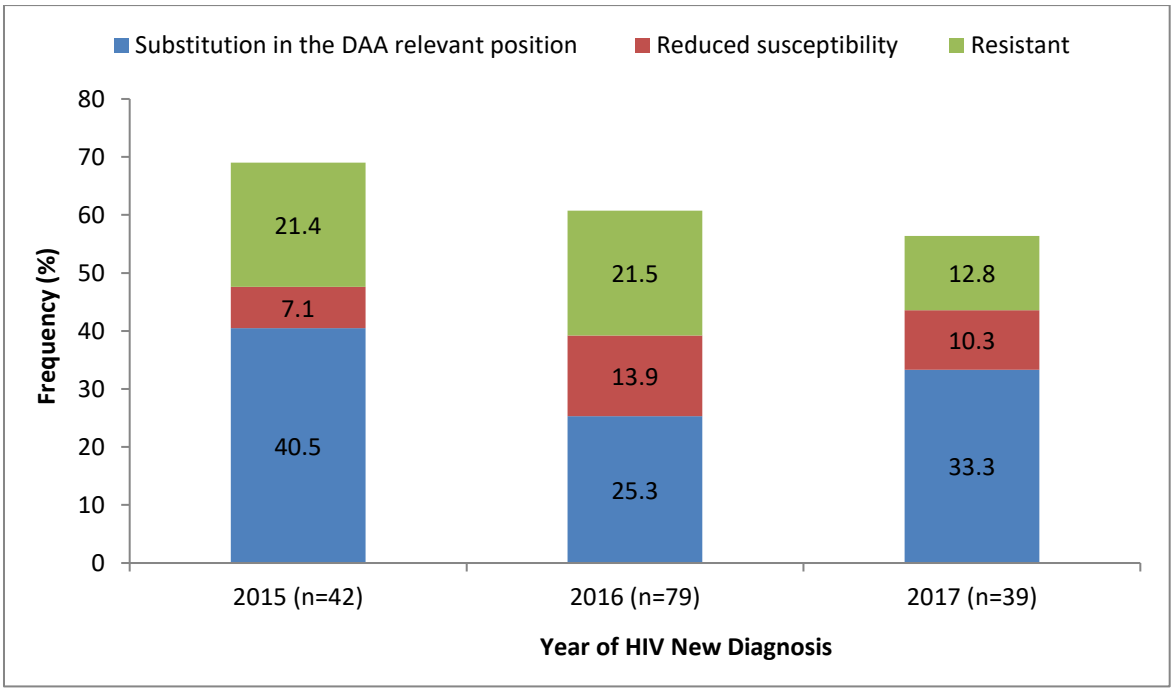


Fig. 46: Overview of the proportions of detected RAS in active HCV infections from the diagnoses years 2015-2017 in relation to all drug classes.

Among the RAS shares in individual HCV subtypes, the highest proportion can be found within subtype 1a (2015: 58.3%, 2016: 79.3%, 2017: 78.6%), subtype 1b (2015: 100%, 2016: 81%, 2017: 75%) and subtype 3a (2015: 77.8 and 2016: 24%) (Fig. 47).

In the subtype 1a samples, 33.3% (2015), 34.5% (2016), 42.9% (2017) of the HCV sequences show resistance at a DAA-relevant position. A high proportion of RAS in the St-1a samples (2016: 25%; 2017: 41.4%; 2018: 35.7%) can only be attributed to "substitutions in a DAA-relevant position." In the case of the subtype 1b, the proportion of substitutions at a DAA-relevant position showed a significant decrease ($p<0.05$) in the diagnoses years 2015 (50%) and 2016 (9.5%), respectively. On the other hand, the proportion of samples whose HCV sequence has a resistance to DAAs was relatively similar throughout the study period (2015: 25%, 2016: 28.6%, 2017: 25%). The proportion of RAS that has a reduced effect on DAAs was highest in the year 2016 (42.9%), compared to the proportions in the diagnoses years

2015 (25%) and 2017 (37.5%). In the subtype 3a samples, a significantly higher ($p<0.001$) proportion of RAS was mainly detected in the diagnosis year 2015 (77.8%) compared with the year 2016 (24%). The RAS in both diagnoses years were due to substitutions at a DAA-relevant position (2015: 55.6% and 2016: 16%, $P<0.01$). There was no RAS detected in the diagnosis year 2017. During the diagnoses years 2015-2016, a decrease ($P=0.16$) in the proportion of resistance to DAAs (16.7% and 4%, respectively) and a slight decrease in RAS that has a reduced effect on DAAs (5.6% and 4%, respectively) were observed. In subtype 4a/d samples, RAS were only detected in the diagnosis year 2016 and were exclusively (50%; n=2/4) substitutions at a DAA-relevant position (Fig. 47).

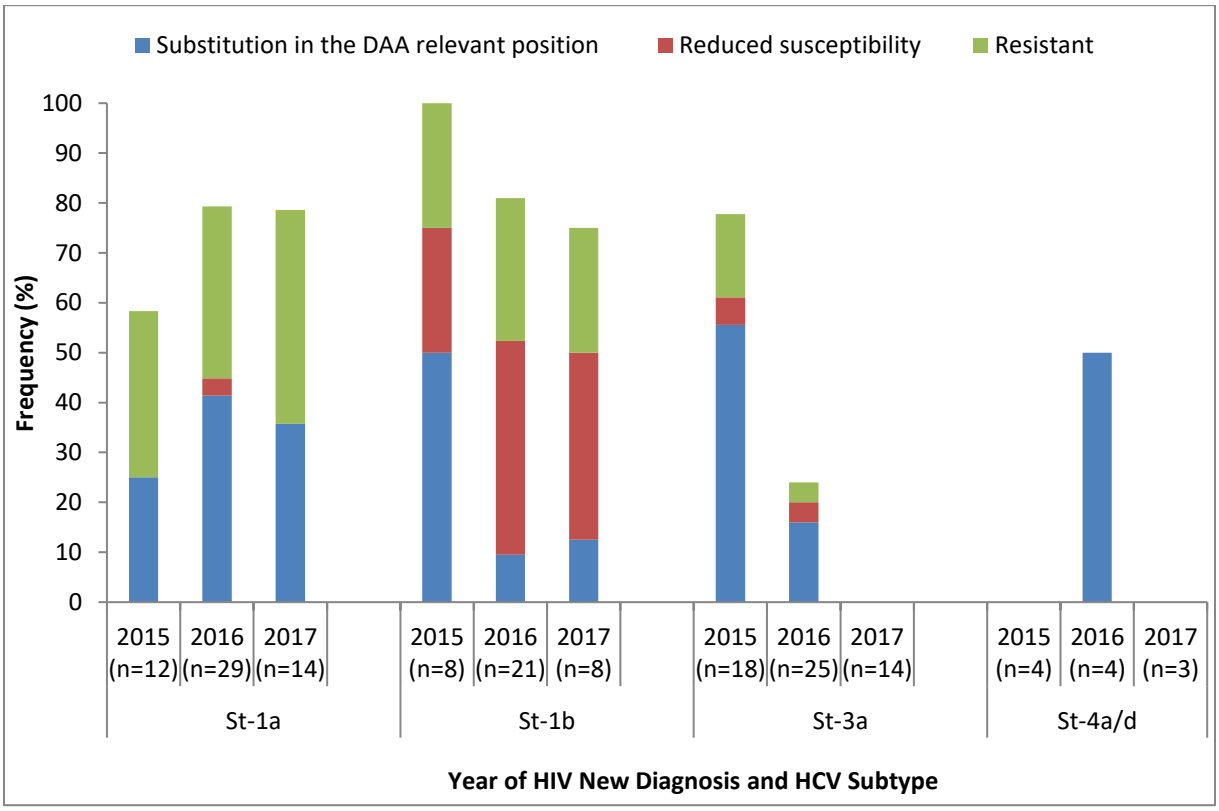


Fig. 47: Overview of the proportions of detected RAS in active HCV infections from the HIV diagnoses years 2015-2017 in the individual HCV subtypes.

The proportion of RAS leading to reduced efficacy or resistance to DAAs in the subtype 1a and subtype 1b samples is high in the diagnosis years 2015-2017, especially in the NS3 region (subtype 1a: 33.3%, 37.9%, 42.9%, respectively and subtype 1b: 37.5%, 38.1%, 25%, respectively) (Fig. 48).

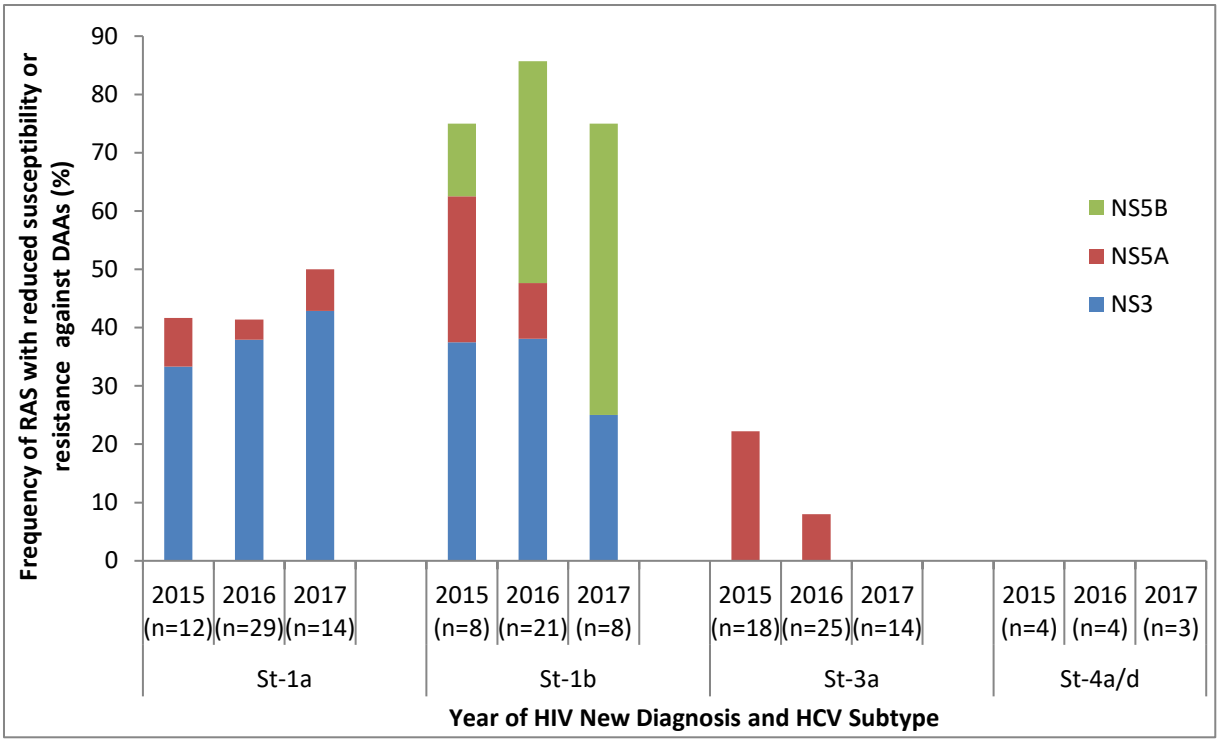


Fig. 48: Overview of the proportions of samples with reduced efficacy or resistance to DAAs in active HCV infections from the HIV diagnoses years 2015-2017 in the individual subtypes.

In the subtype 1a samples, limited efficacy against DAAs is mainly due to polymorphic Q80K substitution in the NS3 region, which favors resistance to the drugs Simeprevir (2015: 33.3%, 2016: 34.5%, 2017: 42.9%) and Voxilaprevir (2015: 33.3%, 2016: 34.5%, 2017: 42.9%) (Fig. 49). RAS in the other genome regions hardly play a role in subtype 1a samples.

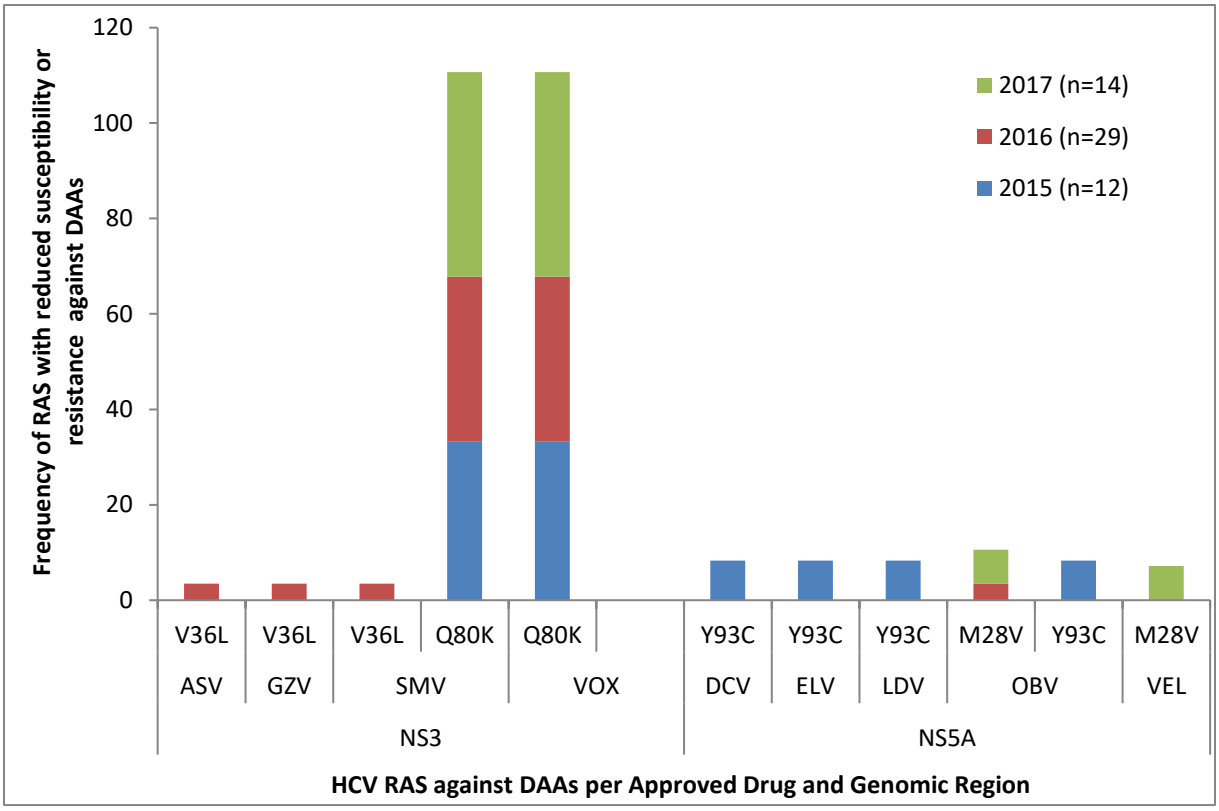


Fig. 49: Proportions of RAS with reduced efficacy or resistance to DAAs per approved drug and genomic regions of HCV subtype 1a samples in the HIV new diagnoses years 2015-2017. ASV= Asunaprevir, GZV= Grazoprevir, SMV= Simeprevir, VOX= Voxilaprevir, DCV= Daclatasvir, ELV= Elbasvir, LDV= Ledipasvir, OBV= Ombitasvir, VEL= Velpatasvir.

In the subtype 1b samples, the reduced efficacy is caused by the Y56F substitution in the NS3 region (2015: 37.5%, 2016: 33.3%, 2017: 25%), which has a reduced efficacy against the drug Grazoprevir (Fig. 50). In addition, there is also an increasing proportion of RAS in the NS5B region, which results in resistance to DAAs (2015: 12.5%, 2016: 38.1%, 2017: 50%) (Fig. 48). This is mainly due to the increasing proportion of S556G substitution in the NS5B region (2015: 0%, 2016: 23.8%, 2017: 25%) conferring resistance to the drug Dasabuvir and L159F substitution in the NS5B region (2015: 12.5%, 2016: 23.8%, 2017: 37.5%), which causes reduced efficacy of the drug Sofosbuvir (Fig. 50). In addition, an increasing and high proportion of the substitution C316N was found (2015: 12.5%; 2017: 42.8%; 2018: 62.5%; data not shown), which alone, however, has hardly any influence on the susceptibility of the DAAs.

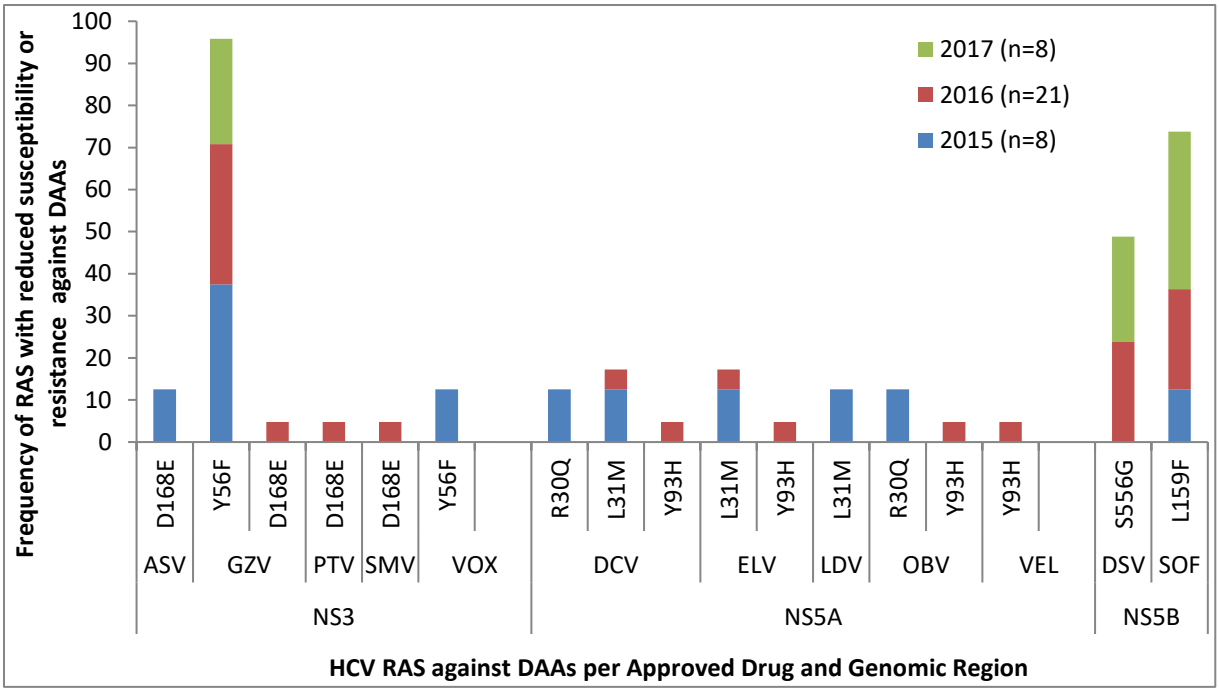


Fig. 50: Proportions of RAS with reduced efficacy or resistance to DAAs per approved drug and genomic regions of HCV subtype 1b samples in the HIV new diagnoses years 2015-2017. ASV= Asunaprevir, GZV= Grazoprevir, PTV=Pibrentasvir, SMV= Simeprevir, VOX= Voxilaprevir, DCV= Daclatasvir, ELV= Elbasvir, LDV= Ledipasvir, OBV= Ombitasvir, VEL= Velpatasvir, DSV=Dasabuvir, SOF=Sofosbuvir.

In the subtype 3a samples, RAS were only detected in the samples from the diagnoses years 2015 and 2016 (77.8% and 24%, respectively; $p<0.001$) (Fig. 48). This is mainly due to the A30K substitution in the NS5A region, which results in reduced efficacy or resistance to all NS5A inhibitors approved for subtype 3a at a similar proportion (2015: 11.1% and 2016: 4%). However, A30K RAS has not been detected in the diagnosis year 2017 (Fig. 51).

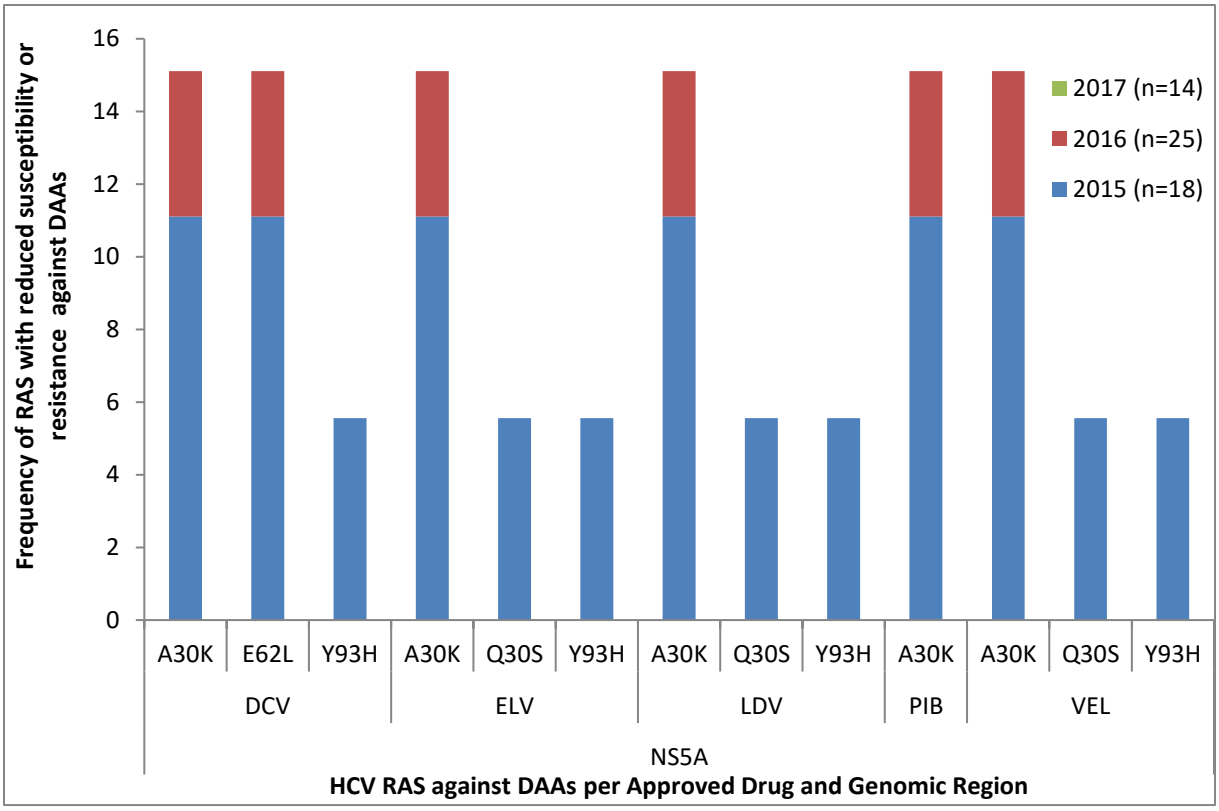


Fig. 51: Proportions of RAS with reduced efficacy or resistance to DAAs per approved drug and genomic regions of HCV subtype 3a samples in the HIV new diagnoses years 2015-2017. DCV= Daclatasvir, ELV= Elbasvir, LDV= Ledipasvir, PIB=Pibrentasvir, VEL= Velpatasvir.

Predominantly, RAS which resulted in reduced efficacy or resistance to DAAs are found in only one genomic region and thus affect the efficacy of only one class of drug. However, especially in the subtype 1b samples, RAS that limit the efficacy of DAAs may also be present in two (2015: 50%, 2016: 6.7% and 2017: 20%) or in all of the three genome regions (2015: 0%, 2016: 6.7% and 2017: 0%) (Fig. 52).

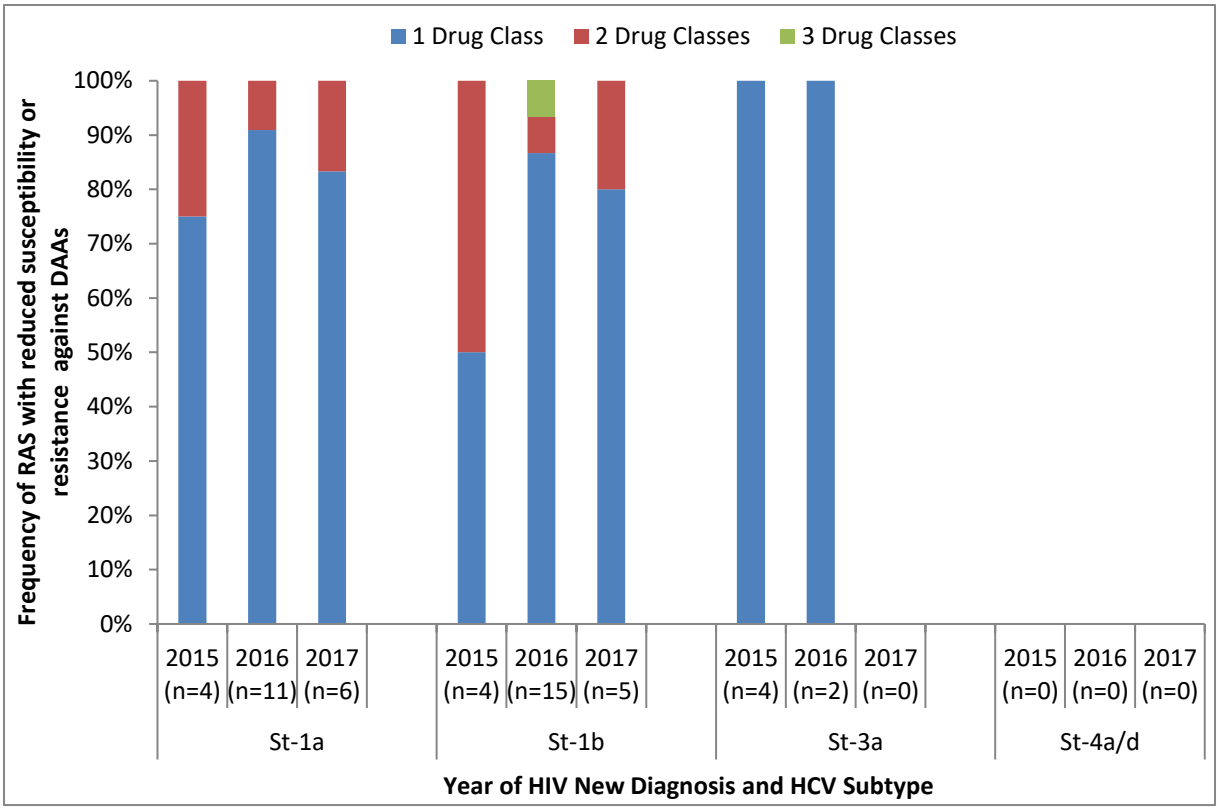


Fig. 52: Overview of the proportions of samples with reduced efficacy or resistance to DAAs in active HCV infections from the HIV diagnoses years 2015-2017 in relation to all drug classes.

5 DISCUSSION

5.1 DISCUSSION (PART I)

A precise and early diagnosis of an acute HCV infection is critical because it provides a chance of cure by antiviral therapy and helps to avoid transmission to uninfected individuals. HCV antigen and antibody combination assays have been developed and introduced as cost-effective alternative methods in addition to nucleic acid testing for early/rapid diagnosis by reducing the window period. This study evaluated the diagnostic performance of Murex and Monolisa antigen/antibody combination assays for DS/PS eluates using plasma and serum as reference samples. In addition, longitudinal HCV panels and clinical DSS eluates were used to screen for previous or current HCV coinfections in newly diagnosed HIV-infected patients.

For the seroconversion plasma panels of subtypes 1a and 2b, the Murex test became reactive for an earlier bleed than the Monolisa test. The results from (Laperche *et al.*, 2015) also demonstrated the relatively better performance of the Murex test in reducing the window period by 45% (about 36.4 days) compared to a reduction by 38% (about 40.5 days) by the Monolisa test. The Monolisa, on the other hand, was relatively more reactive to antibody positive bleeds than the Murex counterpart.

The positive reactivity of the Murex test for the serum samples of subtypes 1b, 3a, and 4d also shows its comparable performance to that of the Monolisa test in the detection of antigen and antibody positive samples. However, for the diluted DSS eluates, the Murex was generally reactive for lower dilutions in the early days of bleed and for almost all dilutions at later time points. The Monolisa test, on the other hand, was reactive to all dilutions across the duration of bleed indicating its superior sensitivity and potential use for HCV screening in DS/PS.

Application of both assays to screen HCV coinfection in DSS eluates of newly diagnosed HIV-cases resulted in a relatively higher proportion of ELISA reactive samples by the Murex test than the Monolisa test. However, as confirmed by RT-qPCR and Western blot, the Murex was found to generate more false-positive results than the Monolisa. The overall correct positive rate of 98.1% in this study by the Monolisa test corresponds to those reported in similar studies (Courouce *et al.*, 2000, Muerhoff *et al.*, 2002, Nubling *et al.*, 2002, Shah *et al.*, 2003, Laperche *et al.*, 2005a, Leary *et al.*, 2006, Odari *et al.*, 2014) pointing towards a better sensitivity and specificity for the use of DSS eluates in HCV screening compared to the Murex. A potential explanation for the higher rate of false positive reactions of the Murex test

with the clinical DSS is a previously reported cross-reactivity with anti-HIV antibodies (Everett *et al.*, 2007).

A limitation in our analysis was that the panels which we used for subtypes 1b, 3a, and 4d were only based on antibody and RNA positive samples. Therefore, the performance of the two assays during the window period (antigen-positive and antibody-negative) was not tested for these subtypes. Moreover, even though regarded highly unlikely, we can formally not rule out that the earlier reactivity of the here tested RNA positive and yet antibody negative samples by the Murex assay is due to a false positive antigen based reactivity.

In conclusion, although we could not intensively test for antigen sensitivity for all HCV subtypes, our results for the plasma samples of subtypes 1a and 2b indicate that the Murex assay performs better for the detection of antigen positive samples, while the Monolisa assay is more suited for antibody detection. On the other hand, the Monolisa assay provides more consistent and reliable results for the detection of different HCV subtypes in dilutions of DS/PS eluates compared to the Murex assay. The Monolisa assay, therefore, indicates its potential use for HCV screening in DS/PS. However, a confirmatory test for Monolisa reactive samples by Western blot and RT-qPCR is recommended in this setting as well. Furthermore, the Murex can serve as an additional diagnostic tool to narrow the window period. Hence, HCV screening strategies involving both DS/PS and a combined antigen-antibody assays could be a method of choice as these tests provide a cheaper and simpler alternative in areas with limited resources.

5.2 DISCUSSION (PART II)

The usefulness of DS/PS to detect viral infections has been established for surveillance purposes (Monleau *et al.*, 2010, Rodriguez-Auad *et al.*, 2015, Hauser *et al.*, 2017, Maldonado-Rodriguez *et al.*, 2017). In this study, for the first time, we have established an in-house antibody-avidity based HCV recency assay for the use of DS/PS in order to distinguish between recent and longstanding infection.

An evaluation panel of longitudinal DS/PS of acute and chronic HCV infections was used to analyze the temporal changes of AIs over time in genotype 1 and non-genotype 1 infections. For both genotypes, stronger antibody avidity is associated with the higher number of days post infection. This is consistent with the findings of the previous studies which were based on whole blood (serum) analysis (Croom *et al.*, 2006, Tuaillon *et al.*, 2010, Hope *et al.*, 2011, Shepherd *et al.*, 2013). There was a sharp increment in AI value for genotype 1 compared to non-genotype 1 infections and this is consistent with the report of Shepherd *et al* (Shepherd *et al.*, 2018).

The FRR of 13.6% (specificity of 86.4%), as calculated from the chronic infection panel (panel 2) for all genotypes at an optimal AI cut-off of 40% and with an estimated duration of <104 weeks, was lower compared to other studies which reported a specificity of 100% at AI cut-off 43% (Gaudy-Graffin *et al.*, 2010) and 99% at AI cut-off of 20% (Shepherd *et al.*, 2013). Furthermore, a prolonged HCV seroconversion has been associated with HIV coinfection (Thomson *et al.*, 2009). Along those lines, a study by Patel *et al* (Patel *et al.*, 2016) reported a specificity of 99% and 92% at an optimal AI cut-off of 20% among HIV-negatives and HIV-positives, respectively. The rather high FRR (low specificity) in our assay evaluation might have been due to the significant proportion (34%) of samples from HIV coinfected individuals in addition to the use of DS/PS instead of serum. Furthermore, differences in the assay characteristics can also be explained by the use of the Monolisa™ Anti-HCV PLUS Version 3 in our study in contrast to the Ortho HCV Version 3 ELISA kit utilized in several previous studies. Dissimilarities in the coated antigen are presumably of paramount importance in this respect.

The FLTR (100-sensitivity) at the optimal AI cut-off 40% and with an estimated duration of <26 weeks was lower for genotype 1 (8.3%; sensitivity of 91.7%), non-genotype 1 (0%; sensitivity of 100%) infections and all genotypes (5.9%; sensitivity of 94.1%) compared to the

sensitivity reported by Shepherd and coworkers (2018) for the same duration of infection at an AI cut-off of 20% (36%, 65%, and 48%, , respectively). The difference could be explained by the different AI cut-off (generally resulting in a lower sensitivity, but higher specificity) and the smaller number of recently infected patient samples in our application panel compared to the above study (n=34 and n=120, respectively).

Patients who cleared the infection after a minimum of 180 days resulted in a decline of AI values over time. Previous studies have also shown lower AI values in these groups of patients compared to patients with chronic HCV infection (Hedman and Seppala, 1988, Kanno and Kazuyama, 2002, Klimashevskaya *et al.*, 2007, Gaudy-Graffin *et al.*, 2010). Moreover, the higher FRR found in this group of patients indicates that this assay can be only applied in viremic patients and it is not useful in HCV RNA-negative samples. This effect was also shown for HIV-recency assays where low viral loads (e.g. following treatment) are used to correct false recent classifications into long-term infections (Kassanjee *et al.*, 2016).

This study has certain limitations. Due to the relatively low number of samples in the sub-panel of longitudinal DS/PS, MDRI across different genotypes was not calculated. Therefore, a larger sample size is required to obtain precise genotype specific MDRI estimates. However, as shown by FRR for genotype 1 infection, the assay performs well for the most prevalent genotype in Germany and can be applied in HCV RNA positive patients. The prolonged HCV seroconversion in HIV coinfected patients and its effect on MDRI estimate, FRR and FLTR should also be analyzed in a size adjusted sample set. It is imperative to note that the assay may not be used in clinical settings. However, its application as a tool for epidemiological studies is in our view of considerable importance.

In conclusion, the described assay and protocol can be used for HCV-recency testing of DS/PS specimens from viremic infections. It performs with an accuracy of 86.9% with an AI cut off of 40%. Below this cut off the sample can be classified as acquired in less than a year with a FRR of 13.6%. At AI values above 40% the sample has to be classified as long standing with a FLTR of 5.9%. An important observation in our study, with relevance to HCV avidity determination in general, is the decline in the AI and the high FRR amongst resolved infections. It indicates that this group could be mistaken for recent infection and that viral load will be a valuable supplementary marker to identify recent infections.

5.3 DISCUSSION (PART III)

This part of the study was the first extensive HCV screening of DSS from newly diagnosed HIV-infected patients in Germany. The aims of this study were to assess the prevalence of HCV infection among risk groups, to determine the HCV genotype/subtype distribution and to identify RAS that are relevant to DAA among newly HIV-diagnosed patients in Germany. For this purpose, 6,097 samples were serologically examined for HCV and the detected HCV infections were further characterized in terms of status (active/resolved), genotype/subtype distribution, DAA drug resistance and their relations with demographic characters.

In the samples tested, a proportion of 6.5% were tested as HCV seropositive. According to the reports of WHO (WHO, 2017), 6.2% of people infected with HIV were additionally infected with HCV worldwide. The proportion of HCV seropositive persons detected in this study is, therefore, similar to the WHO data for the year 2015. Also, in this study, evidence of HCV RNA (active infection) was found in two thirds of HCV seropositive samples (overall 4.2%). In one third (overall 2.3%) antibodies against HCV were present, but no viral RNA was detected (resolved infection). The higher proportion of active infections in this study could be attributed to a less spontaneous clearance and a higher HCV viral load in HIV coinfection (Forns *et al.*, 2017, Asselah *et al.*, 2018).

Between the diagnosis years 2015 and 2016, the proportions of HCV seropositive persons and active infections have non-significantly increased from 6.9% to 7.3% and from 4.2% to 5.4%, respectively. However, a significant decrease in the proportion of HCV seropositive ($p<0.01$) and active (viremic) infections ($p<0.001$) have been observed over the period 2016-2017. This was further demonstrated by a significant increase in the proportion of resolved infections ($p<0.01$) in the period 2016-2017 and in the post-DAA study implying the success of DAA therapy similar to studies elsewhere (Gomaa *et al.*, 2017, Pradat *et al.*, 2018, Iversen *et al.*, 2019).

Although more than half (51.9%) of the participants in this study were MSM, the highest proportion of HCV seropositive cases were observed in the PWID group (77.8%). Also, according to the DRUCK study, 55.5% of HIV-infected PWIDs in Germany are simultaneously infected with HCV (Poethko-Muller *et al.*, 2013). In PWIDs, infections with HIV and HCV are much more common than in the general population due to the sharing of used injectables and the associated high rate of reinfection (Hsieh *et al.*, 2014). In addition,

many of the drug users don't know about their HCV status (Kwiatkowski *et al.*, 2002) indicating that this important transmission group does not benefit so far from the new and efficient HCV treatment options to a similar extent as the other risk groups do. This necessitates more efforts for prevention, testing and treatment in this highly vulnerable part of the population. MSM represent the largest proportion of HIV new diagnoses in this study, but in only 2.5% of the cases could HCV be detected serologically. However, the data obtained here is lower when compared with the results of Jansen *et al* (Jansen *et al.*, 2015) in which case they found 8.2% of the HIV positive MSM to be simultaneously infected with HCV in Germany. The decline observed in the current study could be attributed to the effectiveness of the new interferon-free DAA regimens after the year 2014/2015 in Germany.

Although statistically non-significant, during the study period 2015-2016, an increase in the trend of HCV infection was observed in MSM. This increase parallels a number of other trends including increased sexual risk behaviors including serosorting that includes unprotected sex between men with the same HIV status and the use of “chemsex” drugs, including by injection (Bodsworth *et al.*, 1996, Danta *et al.*, 2008, Schnuriger *et al.*, 2009, Thomson *et al.*, 2009). A further characteristic of the increase in MSM is the alarming number of reinfections reported (Hagan *et al.*, 2015). The increased proportion of HCV cases in PWIDs during the year 2016 (81%) might reflect the increased number of refugees and asylum seekers (Grote, 2018) which had a previous exposure to the virus and later diagnosed in Germany (Jablonka *et al.*, 2017). However, during the year 2016-2017, a decrease in the proportion of HCV infection was noted in all transmission groups. A further reduction in the number of chronic HCV infections can be expected as antiviral treatment options improve (Sarrazin *et al.*, 2012). The higher proportion of resolved infection in all transmission groups, in comparison with the pre-DAA study, reflects the positive impact of such treatments in these groups of patients.

Analysis of recent and longstanding HIV/HCV coinfections using the established HCV recency assay has shown a higher proportion of HCV long-term in HIV long-term infections in all transmission groups and throughout the study period. This is due to the fact that HIV and HCV are transmitted in similar ways and, therefore, people who have HIV may be at higher risk of exposure to HCV (Hsieh *et al.*, 2014). This study has also shown a significant increase ($p<0.05$) in the proportion of HCV recent in HIV recent infections in MSM throughout the study period. Other studies have shown that the incidence of HCV in HIV-

positive MSM is high and it appears to be rapidly increasing over time (van der Helm *et al.*, 2011, Wandeler *et al.*, 2012, Yaphe *et al.*, 2012). Some of the reasons for this case are already mentioned in this part of the discussion.

PWIDs were also observed to have a high proportion (35.5%) of HCV long-term infection in HIV recent cases and this has been almost similar in proportion across the diagnoses years of the study period. This is due to the fact that HCV infection is frequently asymptomatic and many of the PWIDs are not aware of their infection status and, hence, do not perform diagnosis or obtain treatment (Kwiatkowski *et al.*, 2002). The report from WHO (WHO, 2017) also shows that only a minority (20%) of the PWIDs with HCV had been tested and knew their status. On the other hand, earlier clinical detection of HIV compared to HCV due to its shorter latency until manifestations (Kim *et al.*, 2013) could mean early diagnosis and a higher proportion of recent HIV cases in this transmission group.

Among heterosexuals, the highest proportion (33.3%) of HCV recent infection in HIV long-term infection was observed in the study cohort. Furthermore, in this group, the vast majority (74.3%) of the individuals were foreigners who have reported to have acquired the HIV infection in their country of origin (data not shown). Previous studies performed in Germany have also indicated that heterosexual persons who had originated abroad have long-term HIV infection than German natives (Hofmann *et al.*, 2017). Most of these people might have acquired the infection in their country of origin and have later been diagnosed in Germany (Marcus and Starker, 2006). On the other hand, the finding of more recent HCV cases could be attributed to the fact that its transmission is not common in this group of patients. Evidence also shows a lower risk of sexual transmission among heterosexual couples in monogamous relationships (Tohme and Holmberg, 2010). The decrease in the proportion of recent HCV infection in recent HIV cases over the study period could also indicate the more regular and frequent testing of this transmission group over time.

In this study, and also in the pre-DAA study, males contributed to a significantly higher proportion of HCV positive infections (72.8% and 78.3%, respectively). Also, the majority of the HCV coinfected males (78.4%) reported intravenous drug use as a means of transmission. A high prevalence of HIV/HCV coinfection among male PWIDs has been reported in various studies (Solomon *et al.*, 2008, Mahanta *et al.*, 2009, Panda *et al.*, 2014, Saraswati *et al.*, 2015). In females, HCV infection was mainly found in heterosexuals (55.8%) and PWIDs

(44.2%). Other reports also show an association of HCV coinfection with higher rates of anti-HCV antibodies among females engaged in higher-risk sexual practices (Gordon *et al.*, 1992, Rosenberg, 1999, Minola *et al.*, 2006). The increasing proportion of HCV coinfection among female PWIDs across the diagnoses years (2015-2017) could also be due to the overlapping sexual and injecting relationships with male PWID partners for resources such as drugs, injecting equipments and for physical safety and support that makes it more difficult for them to practice safe injecting in such context (Tracy *et al.*, 2014).

The trend in the proportion of resolved infections in males and females follows the trend in the total proportion of resolved infections, with an increase in the post-DAA group ($p=0.17$ and $p<0.05$, respectively) and in the diagnosis year 2017 ($p<0.05$ and $p=0.11$, respectively) which again indicates the benefits of DAA therapy for both sex groups.

The distribution of HCV infection in different age groups shows the highest proportion of infection in the age groups 30-39 (46%) and 40-49 (26.6%). This finding matches with the report of RKI (RKI, 2018) which has also shown a highest peak of infection in these age groups. The finding that these same age groups also have the highest proportion of PWIDs could further indicate the ongoing HCV infection in the middle age groups due to unsafe injection drug use (Mansberg *et al.*, 2018).

In comparison with the pre-DAA study, the proportions of HCV infection in this study showed a significant decline in the age groups 20-24 ($p<0.05$) and 25-29 ($p<0.05$) year olds. In a similar comparison, PWIDs of the same age groups have also shown a similar decrease in the proportion of HCV infection indicating the general decrease of HCV infection among the younger injection drug user population. On the other hand, the increase in the proportion of HCV infection in the age groups of 30-39 and 40-49 year olds points to an ongoing HCV infection in the middle age groups (Mansberg *et al.*, 2018). However, the increase in the proportion of resolved infections in the age groups which have shown a higher proportion of HCV infection can be attributed to successful treatments of HCV in the country (Gomaa *et al.*, 2017, Pradat *et al.*, 2018, Iversen *et al.*, 2019).

Analysis of HCV infection by country of origin has shown that HCV infection is significantly higher in proportion among foreign nationals than Germans and the majority of the HCV infected foreigners are PWIDs who came from the Eastern and Central European regions.

This finding correlates with other studies which also showed HIV/HCV coinfection to be the most prevalent among PWIDs in these regions (Nelson *et al.*, 2011, Platt *et al.*, 2016). A study by Huppe *et al* (Huppe *et al.*, 2008) also showed that, in Germany, immigration from Eastern Europe has contributed to the estimated 23–37% of HCV-infected persons of non-German nationality. These data suggest that targeted screening programs may help in limiting the future health burden in these groups of population (Negro, 2014).

In comparison with the data from the pre-DAA study, there has been an increase in the proportion of HCV infections in the overall foreign (p=0.61), Central Europe (p=0.71), Western Europe (p=0.27) and South/South East Asian (p=0.65) nationals. This indicates that there is still much to be accomplished in enhancing the rates of diagnosis and treatment to prevent disease transmission (Vermehren *et al.*, 2018). However, the gradual decrease of HCV infection combined with the rise in the proportion of resolved infections among most nationalities during the course of the study period (2015-2017) can be attributed to the recent introduction of DAAs which made viral eradication possible in >95% of patients across different populations (Terrault, 2019).

Analysis of HCV genotypes/subtypes has shown mainly subtypes 1a (33.9%), 3a (33.5%), 1b (22.3%) and 4d (3.9%). These subtypes are responsible for the majority of HCV cases worldwide (Weinbaum *et al.*, 2005, Beijer *et al.*, 2012) and in Western Europe (Petruzziello *et al.*, 2016a). The order in the proportion of subtypes remained the same in the pre-DAA study and throughout the diagnoses years of the study period, except for the diagnosis year 2015 where St-3a was the highest (39%) in proportion. It has been shown that the pattern of genotype and subtype distribution can be affected, among other things, by the nature of transmission and migration (Iversen *et al.*, 2019). Therefore, the increased number of refugees and asylum seekers might have lead to this change in the pattern of subtype distribution in the diagnosis year 2015.

When HCV subtypes are analyzed by the country of origin, a significant association ($p<0.01$) of subtype 1a with Germans was observed in this study. This is similar to other studies which also showed a relatively higher prevalence of this subtype in Germans (Berg *et al.*, 1997) and Western Europeans (Robaeys *et al.*, 2016). Moreover, this subtype is significantly associated with MSM ($p<0.01$) and, in comparison with the data from the pre-DAA study, its proportion has shown a non-significant (p=0.54) increase in MSM. A similar high prevalence of subtype

1a was also observed among PWIDs in the pre-DAA study (40.9%) and in this study (33.6%). The high prevalence of subtype 1a among MSM was also observed in a study performed in HIV-negative MSM who started pre-exposure prophylaxis (PrEP) in Amsterdam (Hoornenborg *et al.*, 2017) and in a community-recruited MSM in New York City (Tieu *et al.*, 2018). The association of subtype 1a with PWIDs has also been described in several studies (May *et al.*, 2015b, Kartashev *et al.*, 2016, Robaeys *et al.*, 2016, Petruzziello *et al.*, 2016a).

In the current and pre-DAA studies, a higher proportion of subtype 3a (36.4% and 39.7%, respectively) was found in individuals with foreign origin. The association of subtype 3a with foreign nationals was particularly significant ($p<0.01$) in this study. In addition, both studies have shown highest frequencies (42.9% each) of subtype 3a in the PWIDs and heterosexuals, respectively. A similar predominance of subtype 3a in PWIDs has already been described in Germany and elsewhere (Berg *et al.*, 1997, Petruzziello *et al.*, 2013, Daw *et al.*, 2015, Pawlotsky, 2016, Robaeys *et al.*, 2016). The association of this subtype with foreign nationals and certain transmission groups could indicate its presumed correlation with active immigration from HCV endemic areas.

In the pre-DAA study, subtype 4c was found to be common among individuals of German origin (9.5%). Although genotype 4 is traditionally associated to Central Africa and the Middle East (Mohd *et al.*, 2013, Gower *et al.*, 2014, Petruzziello *et al.*, 2016b), some studies have shown that these genotypes are not uncommon within most European HCV centers (Gower *et al.*, 2014). In this study, subtype 4c was reported to only be found in MSM (20%). This reflects the notion that the transmission of genotype 4 is mainly related to sexual practices, especially in MSM, and in HIV coinfected patients (Cornberg *et al.*, 2011). In the current study, however, this subtype was not found in any of the study participants attributing to the efficiency of the DAAs against genotype 4 infections (Hathorn and Elsharkawy, 2016).

Any amino acid substitution relative to the reference sequence at a position associated with decreased susceptibility to DAAs *in vitro* is generally defined as RAS (Bertoli *et al.*, 2018). In HCV naturally occurring RAS are found with varying degrees of prevalence in the individual genotypes and subtypes. In addition, RAS may be due to the selection pressure of DAAs (Sorbo *et al.*, 2018).

It is not known for the investigated samples whether and to what extent HCV therapy has already been performed. Accordingly, it cannot be assessed whether it is naturally occurring or if it is a drug-selected RAS. However, a comparison of the RAS prevalence in this study (2015: 69%, 2016: 60.8% and 2017: 56.4%) is comparable with a 51.4% of a naturally occurring RAS in HCV sequences of mainly DAA-naive persons in Europe (Chen *et al.*, 2016). The proportion of clinically relevant RAS (i.e. =RAS, which cause reduced efficacy or resistance to DAAs) in the study by Chen *et al* (2016) was 29.3%. The proportion of RAS with clinical relevance found in the samples tested here (2015: 28.5%, 2016: 35.4% and 2017: 23.1%) are, therefore, at a comparable level.

In the diagnoses years 2015, 2016 and 2017, the highest proportion of RAS was detected in genotype 1, especially in the NS3 region (Fig. 42). Frequent Q80K substitution is detected in 4.8% to 75% of all subtype 1a infected individuals and leads to resistance to Simeprevir *in vitro* and *in vivo* (Sarrazin, 2016). The high proportion of samples with the Q80K substitution in our subtype 1a samples in the years of diagnosis 2015 (33.3%), 2016 (34.5%) and 2017 (42.9%) thus meets the expectations. However, Simeprevir has not been available in Germany since May 2018, so the occurrence of this substitution has no effect on the regimen recommended for genotype 1 infections (Sarrazin *et al.*, 2018).

The natural occurrence of C316N and S556G substitution in the NS5B region in subtype 1b is described in the literature with a prevalence of up to 35.6% and 16%, respectively (Sarrazin, 2016). With 12.5%-62.5% (C316N) and 23.8%-25% (S556G), a higher prevalence was found in the samples tested in this study. This could be explained by the unknown proportion of samples from DAA experienced individuals in whom these substitutions were highly selected. Although the C316N substitution alone has little effect on the efficacy of DAAs, the combination with the S556G mutation results in a higher resistance to Dasabuvir than does the S556G substitution alone (Jones *et al.*, 2018). Overall, the data shows that the occurrence of RAS in genotype 1 does not generally lead to treatment failure and SVR is achieved in most cases of DAA initial therapy even without prior resistance testing (Sarrazin *et al.*, 2018).

In genotype 3 and genotype 4, only a few natural RAS are found overall (Sarrazin, 2016). In particular, the Y93H substitution in subtype 3a is associated with a decreased SVR rate (Sarrazin *et al.*, 2018). Although this substitution is detected in the diagnosis year 2015 (5.6%), it hasn’t been detected in the years 2016 and 2017.

In the samples examined, RAS were found predominantly in only one of three DAA-relevant genome regions. Especially in the St-1a and St-1b samples, however, a small proportion of samples were found with RAS in two or all three DAA-relevant genome regions. This can indicate a therapy failure with DAAs, since multiple RAS can be found in St-1a and St-1b samples from DAA therapy failures in particular (Di Maio *et al.*, 2018).

In conclusion, the proportion of HCV coinfections among newly diagnosed HIV cases in Germany is similar to the global coinfection prevalence (6.2%) reported by the WHO (WHO, 2017). In comparison to the pre-DAA study and to the diagnoses years 2015-2016, the overall prevalence of HCV infections have shown a significant decline in the diagnosis year 2017. A further reduction in the number of chronic HCV infections can be expected as antiviral treatment options improve. However, the high prevalence of HCV coinfection in the middle age groups (30-39 and 40-49 years), in PWIDs and in people from Eastern and Central European regions dictates the need to enhance targeted screening programs and treatment to prevent further disease transmission among these groups of population. Furthermore, the increasing rate in the incidence of HCV infection observed in MSM suggests increased sexual risk behaviors which require more extensive risk behavior counseling and routine screening for HCV. Consistent with other reports in Europe (Petruzziello *et al.*, 2016a), this study has found subtype 1a (33.9%) to be the most dominant, followed by subtype 3a (35.5%) and subtype 1b (22.3%) infections. However, the relative prevalence of subtypes changes in relation to the mode of transmission, country of origin and migration. In addition, the high proportion of resistance against DAAs in samples of subtypes 1a and 1b throughout the study period (between 25% and 42.9%) indicates a close surveillance of mutations in this group of patients.

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7 PUBLICATIONS AND PRESENTATIONS

The following publications and presentations resulted from the here-presented work:

A. Articles in international peer-reviewed journals

1. **Eshetu, A.**, Hauser, A., an der Heiden, M., Schmidt, D., Meixenberger, K., Ross, S., Obermeier, M., Ehret, R., Bock, C.T., Bartmeyer, B., Bremer, V., Bannert, N. 2020. Establishment of anti-Hepatitis C virus IgG Avidity test for Dried Serum/Plasma Spots. J Immunol Methods; DOI https://doi.org/10.1016/j.jim.2020.112744.

2. **Eshetu, A.**, Hauser, A., Schmidt, D., Bartmeyer, B., Bremer, V., Obermeier, M., Ehret, R., Volkwein, A., Bock C.T., Bannert, N. 2020. Comparison of two Immunoassays for Concurrent Detection of HCV Antigen and Antibodies among HIV/HCV Co-infected Patients in Dried Serum/Plasma Spots. J Virol Methods; DOI https://doi.org/10.1016/j.jviromet.2020.113839.

3. Wang, B., Kruger, L., Machnowska, P. **Eshetu, A.**, Gunsenheimer-Bartmeyer, B., Bremer, V., Hauser, A., Bannert, N. and Bock, C. T. 2019. Characterization of a hepatitis C virus genotype 1 divergent isolate from an HIV-1 coinfected individual in Germany assigned to a new subtype 1o. Virol J; DOI https://doi.org/10.1186/s12985-019-1135-7.

B. Oral presentations on national meetings

1. **Eshetu, A.,** Bartmeyer, B., Bremer, V., Schmidt, D., Bock, C-T., Bannert, N., Hauser, A. Comparison of two immunoassays for simultaneous detection of HCV Antigen and antibodies among HCV/HIV co-infected patients in dried serum spots. Oral presentation at: ZIBI Graduate School Annual Retreat; 2017 March 16-17; Nauen, Germany.

2. **Eshetu, A.,** Bartmeyer, B., Bremer, V., Schmidt, D., Bock, C-T., Bannert, N., Hauser, A. Comparison of two immunoassays for simultaneous detection of HCV Antigen and antibodies among HCV/HIV co-infected patients in dried serum spots. Oral presentation at: ZIBI Students' Day; 2017 April 04; Berlin, Germany.

C. Poster presentations on national meetings

1. **Eshetu, A.,** Girra, S., Hofmann, A., Bremer, V., Bartmeyer., B., Kücherer, C., Bannert., N., Hauser, A. High Proportion of HIV-1 non-B Subtypes in Lately Diagnosed HIV-Patients. Poster presentation at: ZIBI Graduate School Annual Retreat; 2016 March 17-18; Kremmen, Germany.

2. **Eshetu, A.,** Machnowska, P., Hauser, A., Bock, C.-T., Bartmeyer, B., Schmidt, D., Bremer, V., Bannert, N. Analysis of HCV Coinfections among Newly Diagnosed HIV Cases in Germany Poster presentation at: ZIBI Graduate School Retreat; 2018 April 12-13; Rheinsberg, Germany.

3. **Eshetu, A.,** Girra, S., Hofmann, A., Bremer, V., Bartmeyer., B., Kücherer, C., Bannert., N., Hauser, A. High Proportion of HIV-1 non-B Subtypes in Lately Diagnosed HIV-Patients. Poster presentation at: 26th Annual Meeting of the Society for Virology; 2016 April 06–09; Münster, Germany.

4. **Eshetu, A.,** Bartmeyer, B., Bremer, V., Schmidt, D., Bock, C-T., Bannert, N., Hauser, A. Comparison of two immunoassays for simultaneous detection of HCV Antigen and antibodies among HCV/HIV co-infected patients in dried serum spots. Poster presentation at: 27th Annual Meeting of the Society for Virology; 2017 March 22–25; Marburg, Germany.

5. **Eshetu, A.,** Schmidt, D., Meixenberger, K., Ross, S., Obermeier, M., Ehret, R., Bock, C.-T., Bartmeyer, B., Bremer, V., Bannert, N. and Hauser, A. Anti-Hepatitis C virus (HCV) IgG avidity assay to distinguish between recent and longstanding infections using dried serum or plasma spot. Poster presentation at: 28th Annual Meeting of the Society for Virology (GfV); 2018 March 14-17; Würzburg, Germany.

6. **Eshetu, A.,** Schmidt, D., Meixenberger, K., Ross, S., Obermeier, M., Ehret, R., Bock, C.-T., Bartmeyer, B., Bremer, V., Bannert, N. and Hauser, A. Anti-Hepatitis C virus (HCV) IgG avidity assay to distinguish between recent and longstanding infections using dried serum or plasma spot. Poster presentation at: 29th Annual Meeting of the Society for Virology (GfV); 2019 March 20-23; Düsseldorf, Germany.

D. Poster presentations on international conferences

1. **Eshetu, A.,** Bartmeyer, B., Bremer, V., Schmidt, D., Bock, C-T., Bannert, N., Hauser, A. Comparison of two immunoassays for simultaneous detection of HCV Antigen and antibodies among HCV/HIV co-infected patients in dried serum spots. Poster presentation at: 4th International HIV/Viral Hepatitis Coinfection Meeting; 2017 July 22-23; Paris, France.

2. **Eshetu, A.,** Bartmeyer, B., Bremer, V., Schmidt, D., Bock, C-T., Bannert, N., Hauser, A. Comparison of two immunoassays for simultaneous detection of HCV Antigen and antibodies among HCV/HIV co-infected patients in dried serum spots. Poster presentation at: 9th IAS Conference on HIV Science; 2017 July 23-26; Paris, France.

3. **Eshetu, A.,** Schmidt, D., Meixenberger, K., Ross, S., Obermeier, M., Ehret, R., Bock, C.-T., Bartmeyer, B., Bremer, V., Bannert, N. and Hauser, A. Anti-Hepatitis C virus (HCV) IgG avidity assay to distinguish between recent and longstanding infections using dried serum or plasma spot. Poster presentation at: 5th International HIV/Viral Hepatitis Coinfection Meeting; 2019 July 20-21; Mexico city, Mexico.

4. **Eshetu, A.,** Machnowska, P., Hauser, A., Bock, C.-T., Bartmeyer, B., Schmidt, D., Bremer, V., Bannert, N. Analysis of HCV Coinfections among Newly Diagnosed HIV Cases in Germany. Poster presentation at: 5th International HIV/Viral Hepatitis Coinfection Meeting; 2019 July 20-21; Mexico city, Mexico.

5. **Eshetu, A.,** Schmidt, D., Meixenberger, K., Ross, S., Obermeier, M., Ehret, R., Bock, C.-T., Bartmeyer, B., Bremer, V., Bannert, N. and Hauser, A. Anti-Hepatitis C virus (HCV) IgG avidity assay to distinguish between recent and longstanding infections using dried serum or plasma spot. Poster presentation at: Poster presentation at: 10th IAS Conference on HIV Science; 2017 July 21-24; Mexico city, Mexico.

6. **Eshetu, A.,** Machnowska, P., Hauser, A., Bock, C.-T., Bartmeyer, B., Schmidt, D., Bremer, V., Bannert, N. Analysis of HCV Coinfections among Newly Diagnosed HIV Cases in Germany. Poster presentation at: 10th IAS Conference on HIV Science; 2017 July 21-24; Mexico city, Mexico.

8 DECLARATION

Erklärung

Hiermit erkläre ich, die Dissertation selbstständig und nur unter Verwendung der angegebenen Hilfen und Hilfsmittel angefertigt zu haben.

Ich habe mich anderwärts nicht um einen Doktorgrad beworben und besitze keinen entsprechenden Doktorgrad.

Ich erkläre, dass ich die Dissertation oder Teile davon nicht bereits bei einer anderen wissenschaftlichen Einrichtung eingereicht habe und dass sie dort weder angenommen noch abgelehnt wurde.

Ich erkläre die Kenntnisnahme der dem Verfahren zugrunde liegenden Promotionsordnung der Lebenswissenschaftlichen Fakultät der Humboldt Universität zu Berlin vom 5. März 2015.

Weiterhin erkläre ich, dass keine Zusammenarbeit mit gewerblichen Promotionsbearbeiterinnen/Promotionsberatern stattgefunden hat und dass die Grundsätze der Humboldt-Universität zu Berlin zur Sicherung guter wissenschaftlicher Praxis eingehalten wurden.

Declaration:

I hereby declare that I completed the doctoral thesis independently based on the stated resources and aids.

I have not applied for a doctoral degree elsewhere and do not have a corresponding doctoral degree.

I have not submitted the doctoral thesis, or parts of it, to another academic institution and the thesis has not been accepted or rejected.

I declare that I have acknowledged the Doctoral Degree Regulations which underlie the procedure of the Faculty of Life Sciences of Humboldt-Universität zu Berlin, as amended on 5th March 2015.

Furthermore, I declare that no collaboration with commercial doctoral degree supervisors took place, and that the principles of Humboldt-Universität zu Berlin for ensuring good academic practice were abided by.

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